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The Role Of Thymic Nurse Cells In T Cell Development

By

Deborah D. Philp

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.

2000

Dedicated to my family and in loving memory of
Ilia D. Harris

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/27/00
Date

Chair of Examining Committee
Dr. Jerry Guyden, City College

1/27/00
Date

Executive Officer
Dr. Richard L. Chappell

Dr. Mark Steinberg, City College

Dr. Karen Hubbard, City College

Dr. Howard Petri, Memorial Sloan Kettering

Dr. Ronald Gordon, Mt. Sinai School of Medicine

Supervising Committee

The City University of New York

ABSTRACT

THE ROLE OF THYMIC NURSE CELLS IN T CELL DEVELOPMENT

by

Deborah D. Philp

Advisor: Dr. Jerry C. Guyden

Thymic nurse cells (TNCs) are stromal epithelial cells located in the thymic cortex. A single TNC may contain as many as 200 thymocytes in specialized intra-thymic vacuoles. Very little is known about the process of thymocyte internalization or the phenotypic and molecular changes occurring in developing thymocytes as a function of their interaction with TNCs. The thymocyte-TNC interaction has been difficult to study because TNCs can only be identified and isolated after their interaction with thymocytes in the thymus. Freshly isolated TNCs lack thymocyte internalization capabilities *in vitro*.

The goal of this study was to determine the role played by TNCs in T cell development. The immortalization and development of TNC lines, using SV40 virus (strain PH911) and a temperature sensitive mutant of the SV40 virus (tsA58), provided an ideal model system for studying the function of TNCs in the thymus and their role in T cell development. Previous studies have described functions that are unique to TNCs and have shown that freshly isolated TNCs and immortalized TNC lines possess similar qualities. Electron microscopy and long-term video microscopy were used to show that the thymocyte-TNC interaction occurred via membrane ruffling

and is a selective process. This interactive population displayed a phenotype identical to that of thymocytes prior to MHC restriction; $TCR^{lo}CD4^{+}CD8^{+}CD69^{-}$. A select subpopulation of these thymocytes were rescued from apoptosis as a function of this interaction. The rescued population displayed phenotypic changes characteristic of positive selection and maturation. The resulting thymocyte population displayed a phenotype that is identical to cells that have survived thymic education. The costimulatory effects of the lymphokine IL-1β were required for maturation of the rescued thymocyte population. Blocking studies against MHC class I and class II showed that the MHC-TCR interaction between these two cell types was also required for thymocyte rescue. The non-interactive thymocyte population underwent apoptosis. A population of cells that were not released from the TNCs underwent apoptosis within the TNC's specialized vacuoles. Comparable events were occurring *in vivo* suggesting that TNCs may be involved in selection and play a crucial role in thymic education.

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grandmother, the late Ilia Delita Harris, for encouraging me to reach for the stars. I dedicate this work to my family and to my grandmother's memory.

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INTRODUCTION

T cell development is a multistep process. T cells, like B cells, are bone marrow-derived in origin (Adkins, et.al., 1987). The site chosen for T cell maturation sets these cells apart from other cells of the immune system. The thymus is the primary site of T lymphocyte development and plays a crucial role in the maturation, education and selection of developing lymphocytes. It coordinates the release of competent T cells into the periphery (Paul, 1993). T cell progenitors migrate from the bone marrow to the thymus where thymocyte proliferation and differentiation occur (Ford, et.al., 1966; Le Douarin and Jotereau, 1975; Moore and Owen, 1967). The selective migration of pre-T cells within the thymus during the maturation process has remained a topic of debate. Precursor cells that have the potential to become αβ T cells, γδ T cells, natural killer (NK) cells, and dendritic cells have all been found in the thymus (Anderson, et.al., 1996; Ardavin, et.al., 1993). Potential B cells and macrophages have also been detected in fetal thymocyte cultures (Peault, et.al., 1994). It is within this specialized microenvironment that developing lymphocytes undergo phenotypic changes that are identified by distinct expression patterns of various cell-surface proteins (Miller and Osoba, 1967; Miller, 1994). Positive and negative selection of developing thymocytes also occur during this time. T cell receptor gene rearrangement and the selection process are what shape the organization of the mature T cell receptor. A fraction of these developing T cells will ultimately differentiate into functional cells that will be released from the thymus into the peripheral immune system.

The thymus is a lymphoid organ located in the anterior region of the thoracic cavity (Paul, 1993). It consists of bilaterally symmetrical lobes that are

located at the midline of the body resting on the surface of the pericardium. During the fetal and infant stages of development, the thymus contains many developing T cells embedded in the thymic stroma. This network of epithelium provides the proper microenvironment for T cell development (Janeway, 1996). The thymus is composed of bone marrow-derived macrophages, dendritic cells and non-lymphoid epithelial cells derived from the pharyngeal region (Crouse, Turpen, and Sharp, 1985). Fibroblasts and matrix molecules permeate this entire network. Mesenchymal cells that are of neural crest cell origin (Bockman and Kirby, 1984) surround the thymic primordium. These mesenchymal cells appear to play an important role in development of the thymus. In mice, the thymic rudiment, or thymic anlage, is formed from a combination of ectodermal and endodermal tissue from the third brachial pouch and cleft starting at day 9 of fetal gestation (Weissman, 1985). By day 11 of gestation, the thymic anlage is formed. The ectoderm of the third brachial cleft forms the cortical region and the endoderm of the pouch forms the medulla. The cortex contains more immature thymocytes along with cortical epithelial cells and a few scattered macrophages. The medullary region of the thymus is believed to contain more mature nonproliferating thymocytes, along with dendritic cells, macrophages, medullary epithelial cells and Hassall's corpuscles (Janeway, 1996). While in the cortex, thymocytes are believed to be proliferating (Wekerle, and Ketelson, 1980). When passed into the medulla, cell division is thought to cease and cell maturation begins. When thymocytes travel through the thymus from the cortex to the medulla, they encounter a number of the stromal cell types that are thought to play a part in the development process. During this intrathymic migration, the majority of immature cells are killed before reaching the medulla. Only 1% of the total

thymocyte population survive and become the mature TCR expressing helper ($CD4^+$) and cytotoxic ($CD8^+$) T lymphocytes that are released into the periphery (Andrews, et.al., 1985; Scollay, et.al., 1984). Although the order and duration of these thymocyte-stromal cell interactions are not fully understood, it can be said that these interactions are required for thymic education and the proliferation and maturation of the developing T cell (Haynes, 1984).

Cell proliferation, programmed cell death and alteration of surface protein expression are required processes in T cell ontogeny (Scollay and Godfrey, 1995). The thymus is the major site for the differentiation of mature T cell from their hematopoietic precursors (Miller, 1994; Cantor and Weissman, 1976; Haynes, 1984; Sprent, et.al., 1990). In mice, development in the absence of the thymus would result in a marked lymphocyte deficiency, increased susceptibility to infection and random acceptance of foreign grafts. As a result of the influence received from interactions between thymocytes and the thymic stroma in that specialized thymic microenvironment, antigen receptors that are unique to each mature T cell are produced (Miller, 1967). The diverse T lymphocyte subpopulation found within the thymus suggest that this interactive process is quite complex. Contact between developing thymocytes and thymic epithelial cells is necessary for all stages of thymocyte maturation. If the developing thymus either lacks differentiated thymic epithelium, as seen in nude mice (Pritchard and Micklem, 1973; Nhels, et.al., 1994), or has defective receptor gene recombination, as in SCID mice (Fulop and Phillips, 1989), mature T cells fail to develop.

Mature T cells are divided into two subsets based on their function in an immune response. These subsets are capable of cytotoxic ($CD8^+$) and

helper ($CD4^+$) activity. The possible existence of a third subset that has the ability to suppress or control the immune response has been speculated. However, no suppressor cell clones have ever been isolated. Many cellular immunologists believe that a subpopulation of the $CD8^+$ lymphocytes is responsible for this suppressor function. The existence of more than one functional cell type adds further difficulty to understanding the sequence of the differentiation steps occurring in the thymus. The development of the fluorescence activated cell sorter (FACS) and T cell-specific monoclonal antibodies has supplied the tools necessary to characterize the T cells subpopulations found within the thymus and provided insight into the processes involved in T cell maturation.

Once inside the thymus, developing T cells proliferate and undergo gene rearrangement producing one of two cell lineages: the αβ and γδ T cell antigen receptor (TCR) pathways (Fig. 1).

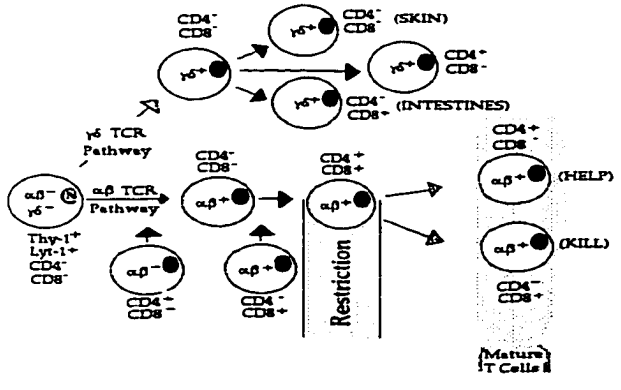

Figure 1. A detailed schematic of cell fate during T cell development in the thymus.

Although the signals required to initiate the decision for these precursor cells to follow either of the two developmental pathways are not yet fully understood, recent studies have shown that expression of Notch gene may act

in conjunction with the newly formed TCR to influence the αβ vs. γδ T cell lineage decision (Washburn, et.al., 1997). These studies imply that reduced Notch expression will favor γδ T cell fate and increased expression will influence T cells to adopt the αβ lineage. γδ cells make up 20% of the entire DN thymocyte population found in the thymus and appear capable of developing in the absence of a functioning thymus. These cells travel through and leave the thymus expressing a DN phenotype. Although the precise function of γδ cells is not known, they home specifically to dendritic epithelial cells of the epidermis and the epithelial layers of the reproductive tract (Haas and Tonegawa, 1992; Itohara, et.al., 1989; Livak, et.al., 1995; Raulet, et. al., 1991). The expression of Notch within the thymus has also been shown to control the fate of developing CD4 and CD8 cells (Robey, 1999). When activated, Notch expression causes a change in the fate of DP cells from a CD4 lineage to a CD8 lineage. This implies that Notch signaling may be regulated by the recognition of MHC class I and II by developing thymocyte receptors.

The studies for this thesis project focus on the αβ TCR developmental pathway. Intrathymic proliferation plays an important role in providing a sufficient number of $\alpha\beta TCR^+$-expressing cells upon which the selection process can operate. Prior to entering the thymus, thymocytes are considered triple negative; they are Thy-1^+CD5^{low} $CD4^-$ $CD8^-$ $CD3^-\alpha\beta TCR^-$ progenitor cells (Fowlkes, et.al., 1985). In mice, immature lymphocytes expressing this phenotype are found in the cortex of the thymus as early as 11 days of gestation (at the same time that the anlage is formed) (Janeway, 1996). These cells also lack the ability to respond to antigen and can not perform effector functions (Li, et.al., 1991). Upon entering the thymus, this phenotype of these cells quickly changes. First, rearrangement of the TCR occurs resulting in

expression of β TCR. Following this, further rearrangement occurs resulting in expression of both CD4 and CD8 along with low level expression of αβ TCR. It is at this point that thymocytes are considered double positive (DP), $CD4^+CD8^+$ intermediate cells. These DN and early DP cells are actively proliferating cells and are located in the thymic cortex. These cells next encounter thymic education. The fate of these cells is determined during this restrictive process. A subset of positively selected $CD4^+CD8^+\alpha\beta TCR^{lo}$ thymocytes appear in the thymus at day 15-16 of gestation (Ohashi, et.al., 1990). A considerable amount of cell death also occurs in the cortical region of the thymus. This death will ensure that only self-tolerant, MHC-restricted T lymphocytes will exit the thymus. The remaining cells migrate through the thymus and will ultimately be released into the periphery as functional helper ($CD4^+$) or cytotoxic ($CD8^+$) T lymphocytes.

The thymic microenvironment is unique and efficient in supporting the development of functional T lymphocytes. The establishment of the T cell repertoire of mature T cells is dependent on the restriction process and self-tolerance (Raulet, 1987). Thymic education is the process by which T cells learn to distinguish what is self and what is foreign (antigen) in the body. The selection process during thymic education is quite intricate and occurs at the DP stage of T cell development. Thymocytes must survive positive and negative selection in order to mature (Kisielow and von Boehmer, 1995). Developing thymocytes recognize self peptide in the context of major histocompatibility complex (MHC) antigen on the surface of thymic epithelial cells, macrophages and dendritic cells (Marrack and Kappler, 1988; Wekerle and Ketelson, Nature, 1980). T cell progenitors have the potential to express TCRs that can recognize both self and foreign antigens associated with any MHC molecule. Positive and negative selection modify the profile of these

cells. Positive selection kills all immature T cells that can not bind to self MHCs that are presenting antigen. Negative selection eliminates potentially autoreactive clones. This will ensure that the mature cell that are released into the periphery are self-tolerant and able to see self in the context of foreign antigen. Understanding the nature of that interaction is considered crucial in thymic education. This process is believed to involve an interaction between the TCR on the surface of the developing thymocyte and the MHC antigen complex on the surface of a thymic epithelial cell in the thymus (Penninger and Wick 1992). The binding affinity between the MHC and the TCR will determine the fate of the developing lymphocyte. The most tested hypothesis proposes that thymocytes producing a TCR that either binds tightly to or lacks the ability to identify self peptide in the context of MHC antigen are not allowed to mature to the single positive phenotype and are selectively deleted. Only those thymocytes that produce a TCR that moderately binds self peptide in the context of MHC are allowed to mature to the single positive phenotype and are released from the thymus as functional T cells. Functional T cells that are foreign antigen specific, self MHC restricted and self tolerant are selected in the thymus. Current immunological studies are focused on identifying the various thymic epithelial cell types and determining their function during restriction.

The study of the interaction between thymocytes and the thymic stroma has greatly enhanced our knowledge of T cell development. In the process of T lymphocyte differentiation, contact between T cell progenitors thymic stromal cells is thought necessary for T cell maturation and thymic education (Wekerle and Ketelson, 1980 Nature; van Vliet, Melis, and van Ewijk, 1984). One cell type believed to be important in the development of T cells is the thymic nurse cell (TNC). TNCs are large, keratin-expressing cells

located in the subcapsular cortical of the thymus (Wekerle, Ketelson and Ernst, 1980-J. Exp. Med.). These cells display a unique relationship with thymocytes. Upon isolation, a single TNC can contain as many as 200 proliferating thymocytes within its cytoplasm. These thymocytes were enclosed in specialized vacuoles. TNCs can be easily differentiated from other cells in the thymus by their unique multicellular structure. This unique cellular complex has been found in a wide variety of species including mice (Wekerle, and Ketelsen, 1980-Nature), rats, goats, sheep, chickens (Boyd, et. al., 1984; Rieker, et. al., 1995) and humans (Ritter, Sauvage and Cotmore, 1981). Since their discovery in 1980 (Wekerle, Ketelson, and Ernst, 1980-J. Exp. Med.), very little information has been reported about TNCs. This is due in part to the fact that these cells are fragile and in limited number ($1X10^5$ cells per thymus) when recovered from the thymus. As a result, very little is known about the interaction between these developing thymocytes and their TNC host or the molecular changes that occur as a function of this event. Also, it has been impossible to study the entire thymocyte/TNC interaction because TNCs can only be identified and isolated following interaction with thymocytes in the thymus. Freshly isolated TNCs do not take up thymocytes *in vitro*.

TNCs express both class I and class II MHC on their surfaces, and on the membranes of the specialized vacuoles surrounding internalized thymocytes (Lorenz and Allen, 1989a). This characteristic is atypical to epithelial cells. TNCs also display the ability to present self antigens to developing T cells (Lorenz and Allen, 1989b). This suggests that TNCs may be involved in thymic education. The process of thymic education is believed to occur at the DP stage of thymocyte development. The MHC molecules are not believed to be involved in the binding and internalization event occurring between

thymocytes and TNCs (Grukowski, et.al., 1985). To date, molecules that mediate the interaction between TNCs and thymocytes have not been defined. These cells have also been shown to express the cortex specific markers ER-TR5 and 6C3 (van Vliet, et. al., 1984; Whitlock, et. al., 1987; Adkins, Tidmarsh and Weissman, 1988).

Focus of Study

The goal of this thesis work has been to determine the functional role of thymic nurse cells (TNCs) in T cell development. In an attempt to increase TNC numbers and viability for study, our laboratory infected freshly isolated TNCs with SV40 virus (Pezzano, et. al., 1991). With this accomplishment, we were able to immortalize cells for future studies and increase the population of TNCs being studied. These studies showed the epithelial nature of TNCs. They also demonstrated that thymocytes were completely internalized by these cells and separated from the microenvironment of the thymus by TNC plasma membrane (Philp, et. al., 1993). TNCs were shown to express both major histocompatibility complex (MHC) class I and class II antigens on their surfaces, but no lymphocyte-specific markers (such as Thy-1, CD5, CD4 or CD8). Studies have suggested that interactions between thymic stromal cells and thymocytes are essential for thymocyte education during T-cell development (Haynes, 1984). That is, the process of learning about self involves specific interactions between MHC proteins on stromal cells and T cell antigen receptor (TCR) on thymocytes. It is believed that self antigen presentation to thymocytes by stromal cells plays an important role in the selection process.

In order to examine thymocyte fate as a function of thymocyte/TNC interaction, cell lines were immortalized with a temperature sensitive

mutant of the SV40 virus, tsA58. The model system used in these studies was a TNC line (tsTNC-14) that has been immortalized with a temperature sensitive mutant of the SV40 virus, tsA58 (Tagmeyer and Ozer, 1971). Previous studies in our laboratory have described some of the functions that are unique to TNCs (Pezzano, et. al., 1991; Li, et. al., 1992). MHC restriction, or thymic education, is involved in positive and negative selection. During this process, the self peptide presenting MHC located on the surface of the antigen presenting cell interacts with the T cell antigen receptor (TCR) on the surface of thymocytes displaying a $\alpha\beta TCR^{lo}CD4^{+}CD8^{+}CD69^{-}$ phenotype (Penninger and Wick, 1992). Positive and negative selection occur based on MHC-TCR complex recognition and binding affinity. TNCs bind and internalize a select subpopulation of thymocytes that display an identical phenotype to those cells involved in MHC restriction. A subset of this interactive thymocyte subpopulation is believed to be apoptotic while the remaining fraction is rescued from programmed cell death. A subset of these rescued thymocytes are believed to be maturing through the process of thymic education. Thymic nurse cells are believed to play a crucial role in this education process. To date, there is an ongoing debate regarding the authenticity, location, and function of TNCs.

Studies have suggested that interaction between thymic stromal cells and thymocytes are essential for thymocyte education during T cell development; that is, the process of learning about self involves specific interactions between MHC proteins on stromal cells and TCR on thymocytes. It is believed that self antigen presentation by stromal cells to thymocytes plays an important role in the selection process (Lorenz and Allen, 1989a). The role of TNCs in the process of MHC restriction is suggested in this study.

The work presented in this thesis has been divided into three chapters. The first chapter (published in the journal Cellular Immunology, 1993), establishes the mechanism involved in the binding and internalization events occurring as a function of the thymocyte/TNC interaction. Phenotypic markers of TNCs were also established as well as the phenotype and viability of the TNC-interactive thymocyte population. The cell line used in these studies (SVT-II2) was immortalized with SV-40 virus. Cultured studies were performed using Newmarski and electron microscopy.

The second chapter is focuses on the ability of TNCs to rescue early $CD4^+CD8^+$ thymocytes from apoptosis. These studies were published in the journal Cellular and Molecular Biology (1995). Thymocyte activation, selection and maturation were all examined with relation to the thymocyte/TNC interaction. The TNC line used in these studies (tsTNC-1), was developed using a temperature-sensitive mutant of the SV-40 virus. MHC class I and class II involvement in thymocyte rescue were also explored.

In the final chapter, thymocyte fate as a function of an encounter with TNCs and the costimulatory effects of IL-1β on thymocyte rescue and maturation were examined (Cellular Immunology, 1996). The cell line tsTNC-1 was used in these cocultures. These studies were performed comparatively with freshly isolated TNCs. FACS analyses were performed on TNC-interactive thymocytes to look for changes in expression of the following markers: Bcl-2, αβTCR, CD69, HSA and PNAr. The occurrence of apoptosis was determined using a modified TUNEL assay. Comparative studies were performed to investigate the necessity of the lymphokine IL-1β for thymocyte maturation and rescue.

CHAPTER 1

Title: **The Binding, Internalization, and Release of Thymocytes by Thymic Nurse Cells**

Condensed Title: Thymocyte Internalization by Thymic Nurse Cells

Authors: Deborah Philp, Mark Pezzano, Yang Li, Coral Omene, William Boto and Jerry Guyden

Department of Biology, The City College of New York, New York, New York 10031, Tel.: (212) 650-8449, Fax: (212) 650-8585

Abstract

Recent studies in our laboratory have described the development of the SV40-transformed thymic nurse cell (TNC) line SVT-II2, that maintains the ability to internalize thymocytes *in vitro*. SVT-II2 cells were shown to bind and internalize a subset of the αβ TCR^+, $CD4^+CD8^+$ thymocyte population exclusively. Also, SVT-II2 cells express cell surface class I and class II MHC antigens. These data are consistent with reports that suggest TNCs may have a role in thymic education. In this report, we used scanning electron microscopy, transmission electron microscopy, and long term video microscopy to study binding, internalization, and release of thymocytes by TNCs. The results of these experiments showed the internalization event to be selective and dynamic. The process appears to involve programmed

cooperation between the two cell types that terminates with the release of selected thymocytes. Although no changes in thymocyte cell surface phenotype were detected as a result of their interaction with TNCs *in vitro*, 4-11% of the internalized cells was selectively killed.

1. *Abbreviations used:* TNC, thymic nurse cell; SVT-II2, an SV40-immortalized thymic nurse cell line; TCR, T cell antigen receptor; MHC, major histocompatibility complex.

Introduction

Very few mammalian cells have the capacity to internalize other mammalian cells. The *in vitro* endocytosis of mammalian cells has been described for macrophages only (Aggeler and Werb, 1982). Damaged or senescent cells are selectively taken up and destroyed by this phagocyte in a receptor mediated fashion (Griffin et al., 1976). Sertoli cells are believed to take up immature sperm cells during spermatogenesis (Russell, 1980). The relationship between Sertoli cells and developing sperm cells is presently being studied (Russell and Peterson, 1985). Thymic nurse cells take up thymocytes, but the purpose of their uptake and the internalization mechanism are unknown. This is the first report describing the mechanism employed by TNCs to establish their multicellular structure.

For over ten years scientists have been limited to only descriptive analyses of thymic nurse cells (TNCs) because it was difficult to obtain the large number of cells needed for their extensive examination. Thymic nurse cells contain from 20-200 dividing lymphocytes within their cytoplasmic

membrane (Wekerle and Ketelson, 1980; Wekerle et al., 1980). Thymocytes in this complex are trypsin insensitive, and electron microscopic studies show them to be completely enclosed within the TNC membrane vacuoles. Because of their multicellular nature, TNCs can be experimentally separated from other cells of the thymus as a function of their density (Wekerle et al., 1980; Kyewski et al., 1982). However, the maximum recovery efficiency per mouse thymus is only 10^5 cells using the density gradient method. There are two additional problems with the use of this isolation procedure. First, it is impossible to definitively determine the phenotype of internalized thymocytes because unenclosed thymocytes contaminate the TNC fraction. Secondly, the initial interaction between TNCs and their resident thymocytes must occur before their isolation if the density gradient procedure is to be exploited, making an analysis of the formation of this unique cell-to-cell complex impossible. In an attempt to improve experimental procedures for immunological studies of the role of TNCs in thymocyte development, we immortalized TNCs using the SV40 virus, and developed several cell lines (Pezzano et al., 1991). Our recent reports verified that the thymic epithelial cells immortalized by the SV40 virus were nurse cells, and showed the thymocytes that interact with TNCs to express both CD4 and CD8 on their cell surfaces (Pezzano et al., 1991; Li et al., 1992). Fortunately, these immortalized TNCs maintained the ability to internalize bound double positive lymphocytes. Here, we used various microscopic procedures to show the actual uptake of thymocytes by TNCs. Bound thymocytes were shown to produce filamentous projections for attachment to the external surface of the TNC plasma membrane. TNC membrane ruffling resulted in waves of moving membrane. Attached thymocytes moved along with the membrane, and internalization was initiated when the leading edge of the wave of

membrane moved over bound thymocytes from one side. The TNC membrane then fused to itself after covering trapped thymocytes. Thymocyte movement continued immediately after entry into the TNC. This movement is believed to be directionally controlled because cytoplasmic channels became visible within the TNC simultaneous to the initiation of internalization. Internalized thymocytes were shown to move into and within these channels a short time after their uptake.

Thymic education is believed to occur at the double positive stage of T cell development (Marrack and Kappler, 1988; Schwartz, 1989). The binding specificity of TNCs for a subset of the $CD4^+CD8^+$ thymocyte population suggests that this interaction may play a role in thymic education. The process of thymic education 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 epithelial cells. 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 (Wekerle and Ketelson, 1980; De Waal Maleifijt et al., 1986). Further, Lorenz and Allen (1989 a, 1989 b) reported TNCs to have antigen presenting capabilities. In this study, specialized contact structures are shown to form between internalized thymocytes and TNCs within cytoplasmic vacuoles *in vitro*. Membrane contact is required to facilitate the TCR-MHC interaction. We also show selected thymocytes to be released into the extracellular environment. Although cell surface changes were not detected within the internalized thymocyte population over the life of these cells in culture, 4-11 percent of internalized cells are selectively killed within vacuoles.

Materials and Methods

Cells . 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 exposed to monolayers of the SV40-immortalized TNC line termed SVT-II2 (Li et al., 1992). $2X10^6$ thymocytes were plated in 35 mm tissue culture dishes containing a monolayer of SVT-II2 cells (initial concentration of TNCs was $1X10^4$ 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 antibodies made against CD4 (an anti-CD4-phycoerythrin (PE) conjugate) and CD8 (an anti-CD8-fluorescein (FITC) conjugate). Both of these antibodies were obtained from Boehringer Mannheim. The stained cells were analyzed using a Zeiss fluorescence microscope equipped with epi-illumination. Viability of thymocytes was assayed using propidium iodide (5 μg/ml). Propidium iodide was incubated with TNCs containing bound or cytoplasmic thymocytes for 30 minutes at room temperature. Each sample was washed five times before viewing with the Zeiss fluorescence microscope.

Long Term Video Microscopy. Thymocytes co-incubated with SVT-II2 cells as described above were viewed using a Nikon Diaphat Inverted Microscope with a Hoffman Modulation Contrast System. The microscope was attached to a Nikon CCD-72 camera. The samples were visualized on a Sony 19 inch

color monitor coupled to a JVC 1/2 inch VCR. The tissue culture flasks were bubbled with CO_2 and capped tightly. The microscopy was performed in a warm room at 37°C.

Transmission Electron Microscopy. Thymocytes were co-incubated with SVT-II2 cells for 1, 2, 4, 5 and 6 hours. Unbound thymocytes were removed by washing 3X with PBS. TNCs were fixed with 2% gluteraldehyde made in 1X PBS for 1 hour at 4°C. The cells were then washed twice with 1X PBS, and treated with the secondary fixative, 1% osmium tetroxide, at room temperature for 30 minutes. The cells were rewashed in 1X PBS, removed from their surface and pelleted. Dehydration steps were done in ascending percentages of ethyl alcohol for five minutes each terminating in three 10 minute treatments in 100% ethyl alcohol. A 1:1 dilution of 100% ethyl alcohol and propylene oxide was added to the pellet for 10 minutes, followed by a 30 minute incubation in 100% propylene oxide at room temperature. The cell pellets were then transferred to beam capsules and incubated in a 1:1 dilution of propylene oxide and epon 812 resin for 1 hour at room temperature followed by an overnight incubation in 100% resin mixed with the polymerizer DMP-30. Each specimen block was then polymerized at 65°C overnight.

The specimen block was mounted in the LKB MT-2 microtome and sections were obtained using a blade angle of approximately 3°. Ultra-thin sections were collected using copper grids that were pre-washed in acetone. The sections were stained using a heavy metal double staining technique with 5% uranyl acetate and lead citrate. The grids were viewed using the Phillips 300 Transmission Electron Microscope at a voltage of 60 V and photographs were taken using Technical Pan film.

Scanning Electron Microscopy. Again, thymocytes were co-incubated with SVT-II2 cells for 1, 2, 4, 5 and 6 hours. Cell samples were taken and fixed at 4°C for one hour in 2% gluteraldehyde made in 1X PBS. Washes were done twice in 1X PBS prior to a 30 minute incubation of the cells in the secondary fixative, 1% osmium tetroxide. Each plate was washed 3 times with 1X PBS and then dehydrated in ascending percentages of ethyl alcohol at 10 minutes each. Three treatments with 100% ethyl alcohol were done immediately followed by critical point drying using a CPD 030 critical point dryer. The samples were mounted on stubs using silver paint and dried overnight at 60°C. The stubs were then sputter coated in gold at a thickness of 10 nm. A Zeiss Scanning Electron Microscope, at 15 kv, was used to view samples. Photographs were taken using Kodak 4415 film.

Results

Binding and Internalization of Thymocytes by TNCs

In an attempt to visualize the dynamics of the process of internalization, thymocytes bound to a monolayer of the SV40-transformed TNC line, SVT-II2, were analyzed using long term video microscopy (Fig. 1). Photographs taken from the video monitor show the internalization of the thymocyte marked X. Each photograph, from left to right, represents an 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. 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). Internalization is completed in frame 822. The internalized thymocyte (X) moves under the thymocytes labeled 1, 2, and 3, (these three thymocytes remain bound to the external cell surface of the TNC, frames 822, 835, 853) and into the preformed channel (frame 873, arrows). The internalized thymocyte continues to move in a directional manner within the channel (frames 889 and 897). Much higher magnifications are needed to determine the TNC membrane activity during the internalization event. To visualize this process at higher magnifications, thymocytes were exposed to monolayers of SVT-II2 cells and prepared for scanning electron microscopy after incubation periods of 1, 2, 4, 5, and 6 hours. Thymocyte binding was detected after the initial one hour incubation period (Fig. 2A, B, and C). Thymocytes bound to the external surface of the TNC plasma membrane produce finger like projections that appear to aid in their attachment. Thymocytes are shown to move around the surface of the TNC as a function of their attachment to the TNC membrane (Fig. 2D and E, arrows, and Fig. 4, frames 3743-4108, cells labeled A and B). The bound thymocytes appear to be carried along with a wave of TNC membrane (Fig. 2D and E, arrows) as it slides over itself producing layers. Thymocyte internalization begins when the leading edge of the sliding TNC membrane moves over the top of bound thymocytes (Fig. 2F). The TNC membrane moves above attached cells from one side. After completely covering trapped thymocytes, the TNC membrane fuses to itself in an area on the other side of the cells being internalized. Attachment and fusion points are shown in Figure 2F (arrows). The initiation of the internalization process was detected in samples taken at 2 hours (Fig. 2D, E, F). At 4-6 hours many thymocytes

were completely internalized (Fig. 2G-O). Internalization was shown to be specific to a subpopulation of bound thymocytes because some cells were shown to be selectively excluded from the process (Fig. 2H, arrows). It should be noted that the TNC membrane above and below the trapped thymocytes displayed different characteristics in the 4 hour sample (Fig. 2H and I). The membrane beneath (labeled b in Fig. 2H and I) the thymocytes had many microvilli, but the membrane above these cells (labeled a in Fig. 2H and I) was smooth. These surfaces are different membrane areas of the same TNC. At 6 hours, a second layer of TNC membrane was shown to move over previously trapped thymocytes (Fig. 2N and O, arrows).

The Interactions between the Internalized Thymocytes and the Cytoplasm of TNCs

To obtain a cytoplasmic view of thymocytes after internalization, cells forming the TNC-thymocyte complex were sectioned and prepared for transmission electron microscopy. Micrographs show TNC vacuoles to contain one (Fig. 3B, C, and D) to several (Fig. 3E-H) thymocytes. Several specialized contact structures were detected between membranes of enclosed thymocytes and TNCs (Figures 3D, and inserts 3F and 3H). Contact between individual thymocytes within the vacuole is shown in Figure 3G. Time lapse video microscopy showed thymocytes within these multi-populated vacuoles to move around each other in a programmed fashion (Fig. 4). That is, the thymocytes labeled 1, 2, 3, 4 and 5 moved within the vacuole in a circular pattern before lining up with other internalized cells. The purpose of this type of thymocyte movement, and the cellular machinery responsible for this unusual activity are unknown.

Thymocyte Release

Release of an internalized thymocyte is demonstrated in Figure 5. The thymocyte under investigation is marked with an X. To show that the TNC membrane separates the internalized thymocytes from those bound to the external surface, thymocyte X can be seen moving under two externally bound thymocytes, labeled 1 and 2, to the opposite side of these cells (frames 0007-0067). The internalized thymocyte travels toward the edge of the TNC, and is eventually released. Complete separation of the thymocyte from the TNC can be seen in frame 0744 (arrows). Although release of thymocytes can be visualized after thorough analysis of video tape, it is impractical to isolate released thymocytes away from other cells in these cultures in the quantities necessary for analyses. In an attempt to determine the effect of TNC internalization on thymocyte expression of CD4 and CD8, internalized cells were stained with antibodies made against CD4 and CD8 after incubation periods of 12, 24, 36 and 48 hours. In an earlier report, we showed TNCs to exclusively bind and internalize a subset of immature αβ TCR^+, $CD4^+CD8^+$ thymocytes (Li et al., 1992). Here, we determined the cell surface phenotype of internalized thymocytes over the life of these cells in culture (Table IA). 95-96% of internalized thymocytes were double positive. No CD4 nor CD8 single positive thymocytes were detected in the internalized population over the period tested. The internalized cells that did not stain with either CD4 or CD8 (4-5%) appeared dead. Unstained thymocytes displayed irregular shapes with deformed nuclei. In similar experiments, we used propidium iodide to distinguish between live and dead thymocytes after internalization (Fig. 6). Although dead thymocytes do not bind TNCs, between 4-11 percent of

internalized cells stained brightly with propidium iodide (Table IB) during the 48 hour incubation period.

Discussion

Rarely do mammalian cells pass through the cytoplasm of another cell during their development. Sertoli cells and thymic nurse cells are the only mammalian cell types known to produce structures that completely enclose developing cells within cytoplasmic compartments. Details of spermatogonia uptake by Sertoli cells have not been reported. However, in this study we have described the process used by TNCs to internalize immature thymocytes. We showed thymocyte binding to be essential but insufficient for internalization to occur. Our earlier studies showed TNC binding to be specific for a subset of the αβ TCR^+, $CD4^+CD8^+$ thymocyte population (Li et al., 1992). The binding specificity of TNCs for this double positive subset suggests that the interaction is receptor mediated, although the molecular components involved have not been identified. Movement of bound thymocytes appears to be facilitated through their attachment to the TNC plasma membrane. The TNC plasma membrane is very dynamic and attached thymocytes are pushed or pulled along its surface via membrane ruffling. TNC membrane ruffling creates waves in the lipid bilayer that move along the surface of the cell (Fig. 7). The cellular mechanism used to generate TNC membrane movement is unknown. Internalization is initiated when the leading edge of the ruffled membrane slides over bound thymocytes. We believe that the structure formed around these thymocytes creates a channel to facilitate their movement within the TNC. The

cytoplasmic structures involved in channel formation have not been identified.

Transmission electron microscopic studies revealed that internalized cells reside in vacuoles. These vacuoles contain one to several thymocytes. We have not determined if multi-populated vacuoles result from the fusion of many vacuoles, each containing a single thymocyte, or from the internalization of several thymocytes into one vacuole. Our microscopic studies have produced evidence for the occurrence of both events. Specialized contact structures develop between the membrane of the internalized thymocyte and that of the vacuole surrounding it. Contact is important if this interaction plays a role in thymic education. Penninger and Wick (1992) have recently shown TNC thymocytes to have self-MHC reactivity. These data along with experiments that show TNCs to have the ability to present antigen (Lorenz and Allen, 1989 a, b) suggest that they may have a function in MHC restriction. However, is internalization of thymocytes required for this selective process to occur? We showed binding of the TCR to the self-MHC complex not to be a requirement for internalization to occur (Li et al., 1992). The experiments by Lorenz and Allen (1989) showing TNCs to have the capacity to present antigen did not require thymocyte internalization. Also, earlier reports showed TNCs to express both class I and class II MHC antigens on their plasma membrane as well as on the membranes forming their specialized cytoplasmic vacuoles (Wekerle and Ketelson, 1980; De Waal Malefijt et al., 1986). It is possible for the interaction between the MHC antigens expressed on the cell surface of TNCs and the TCR on the surface of thymocytes to occur in the absence of internalization. What then is the function of the internalization event? One obvious but hypothetical explanation for the function of thymocyte internalization is that

the specialized TNC vacuole provides a compartment that is intimately associated with the cytoplasm of the TNC which produces a stable microenvironment for the presentation of a large number of self antigens to selectively enclosed thymocytes. Positive selection of internalized thymocytes would result in their release to continue the developmental process, while thymocytes selected against would be killed. In this study, TNCs were shown to have the ability to selectively release or destroy internalized thymocytes. The thymocytes involved in this interaction maintain their double positive phenotype throughout the co-incubation period. These data are consistent with studies showing changes from the $CD4^+CD8^+$ phenotype to either CD4 or CD8 single positives to occur only after thymocyte interaction with stromal cells of the medulla (Teh et al., 1988; Hugo and Potworowski, 1990; Ohashi et al., 1990). TNCs are cortical cells and their interaction with thymocytes is believed to precede that of medullary epithelial cells in the T cell developmental pathway (Kyewski et al., 1989; reviews, Spent et al., 1988; Fowlkes and Pardoll, 1989). Also, it has been proposed that negative selection occurs within two thymocyte subpopulations. Cells that do not recognize self at all, and cells that strongly recognize self are deleted (reviewed in Kourilsky and Claverie, 1989; Schwartz, 1989). The cytoplasmic vacuoles of TNCs are ideal structures to provide the proper microenvironment for the identification, isolation, and elimination of unwanted cells.

Although much work remains before TNC function is defined, these data strongly suggest that selected thymocytes are completely enclosed within the thymic nurse cell cytoplasmic membrane. Much debate has been generated over the last decade about the nature of the interaction between TNCs and thymocytes. It has been difficult to definitively demonstrate that the structure surrounding thymocytes is the membrane of a cell. It has been

suggested that the multicellular structures, termed thymic nurse cells, are aggregates of cells produced from incomplete enzymatic digestion during the isolation procedure. In the experiments reported here, cells from a TNC line, SVT-II2, are maintained in culture devoid of cytoplasmic thymocytes. The multicellular structure characteristic of TNCs develops only after exposure of these cells to freshly isolated thymocytes. Furthermore, we conclusively show trapped thymocytes to be completely membrane enclosed after the internalization event.

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FIGURES AND LEGENDS

Fig. 1. The Dynamics of Thymocyte Internalization. Freshly isolated thymocytes were added to monolayers of SVT-II2 cells and incubated overnight. Unattached thymocytes were removed by washing, and the resulting cultures 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 1/2 inch VCR. The thymocytes labeled 1, 2 and 3 are bound to the external surface of a TNC. The nucleus of the TNC is labeled N. The thymocyte labeled X is internalized in the series of micrographs. The arrows show a cytoplasmic channel that forms before the initiation of internalization.

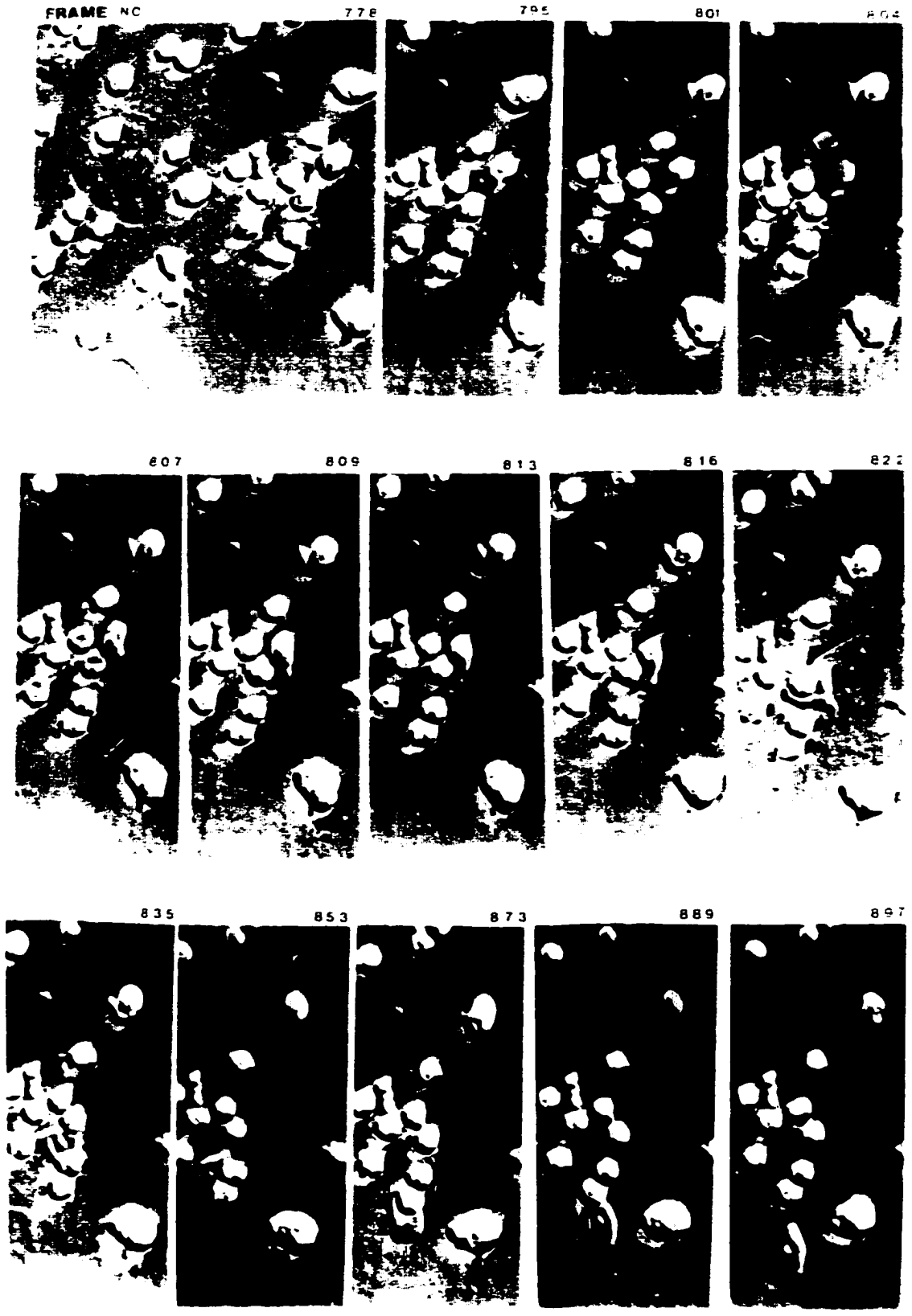


Fig. 2. SEM of Thymocyte Internalization. Thymocytes were centrifuged onto monolayers of SVT-II2 cells and prepared for scanning electron microscopy after incubation periods of 1 hour (A-C), 2 hours (D-F), 4 hours (G-I), 5 hours (J-L), and 6 hours (M-O). Arrows (D and E) show a wave of TNC membrane attached to bound thymocytes. Arrows (F) show attachment and fusion of the TNC cytoplasmic membrane after trapping bound thymocytes. Arrows (H) show bound thymocytes that are selectively excluded from the internalization process. In Figures H and I the TNC membrane above trapped thymocytes is labeled (a), and the TNC beneath trapped thymocytes is labeled (b). Arrows (N and O) show a second layer of TNC membrane covering internalized thymocytes.

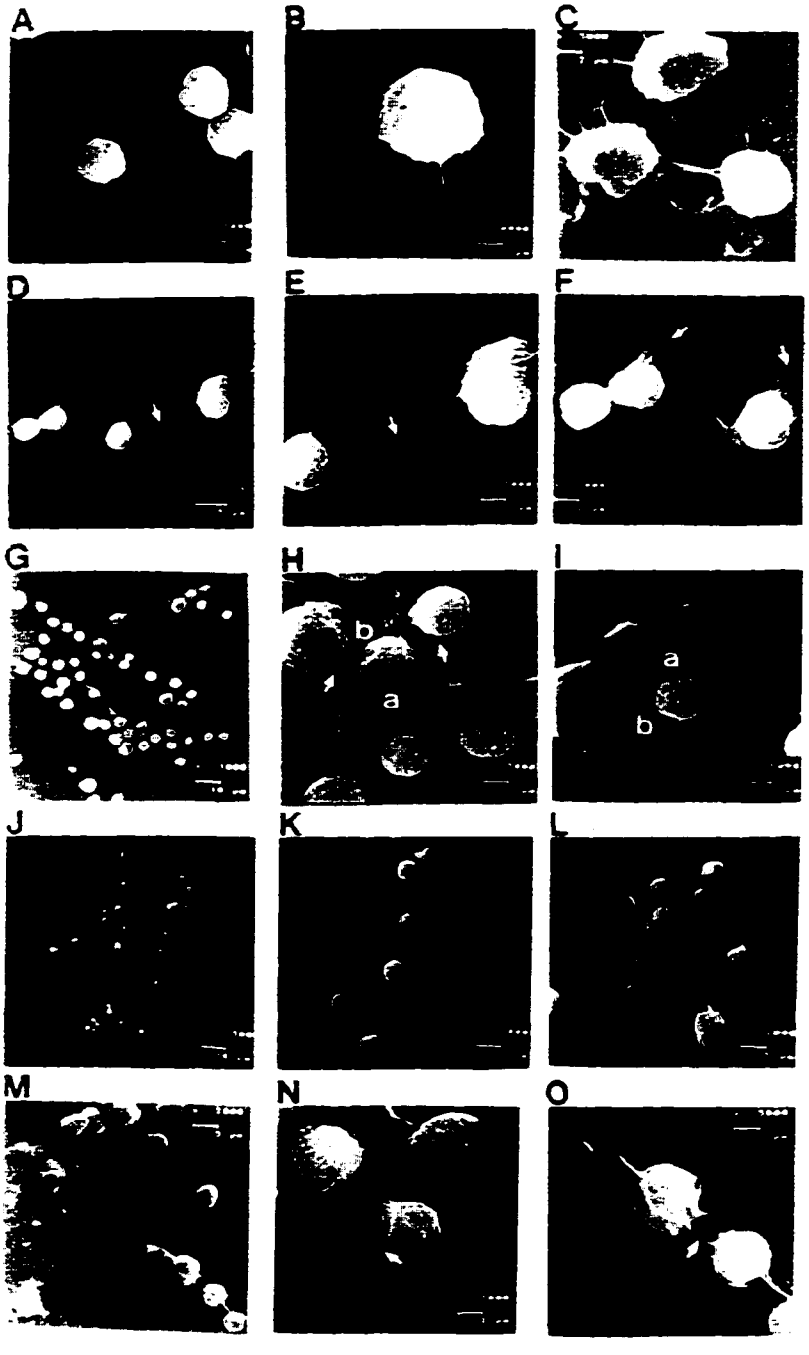


Fig. 3. TEM of Internalized Thymocytes. Thymocytes were centrifuged onto monolayers of SVT-II2 cells and incubated overnight before being prepared for transmission electron microscopy. (A) An SVT-II2 TNC before exposure to thymocytes. (B-D) Micrographs of a TNC containing one internalized thymocyte at increasing magnifications. Magnifications (A) $6.8X10^6$ (B) $3.3X10^3$, (C) $5.6X10^3$, and (D) 10^4. Another thymocyte can be seen outside of the TNC cytoplasm. (E-H) Micrographs of a TNC containing three internalized thymocytes at increasing magnifications. Magnifications (E) $3.9X10^3$, (F) $4.6X10^3$, (G) $5.6X10^3$, and (H) $8.2X10^3$. Inserts in G and H show specialized contact structures between the internalized thymocytes and the TNC. (c) Contact points between thymocytes. (In) Area shown in insert.

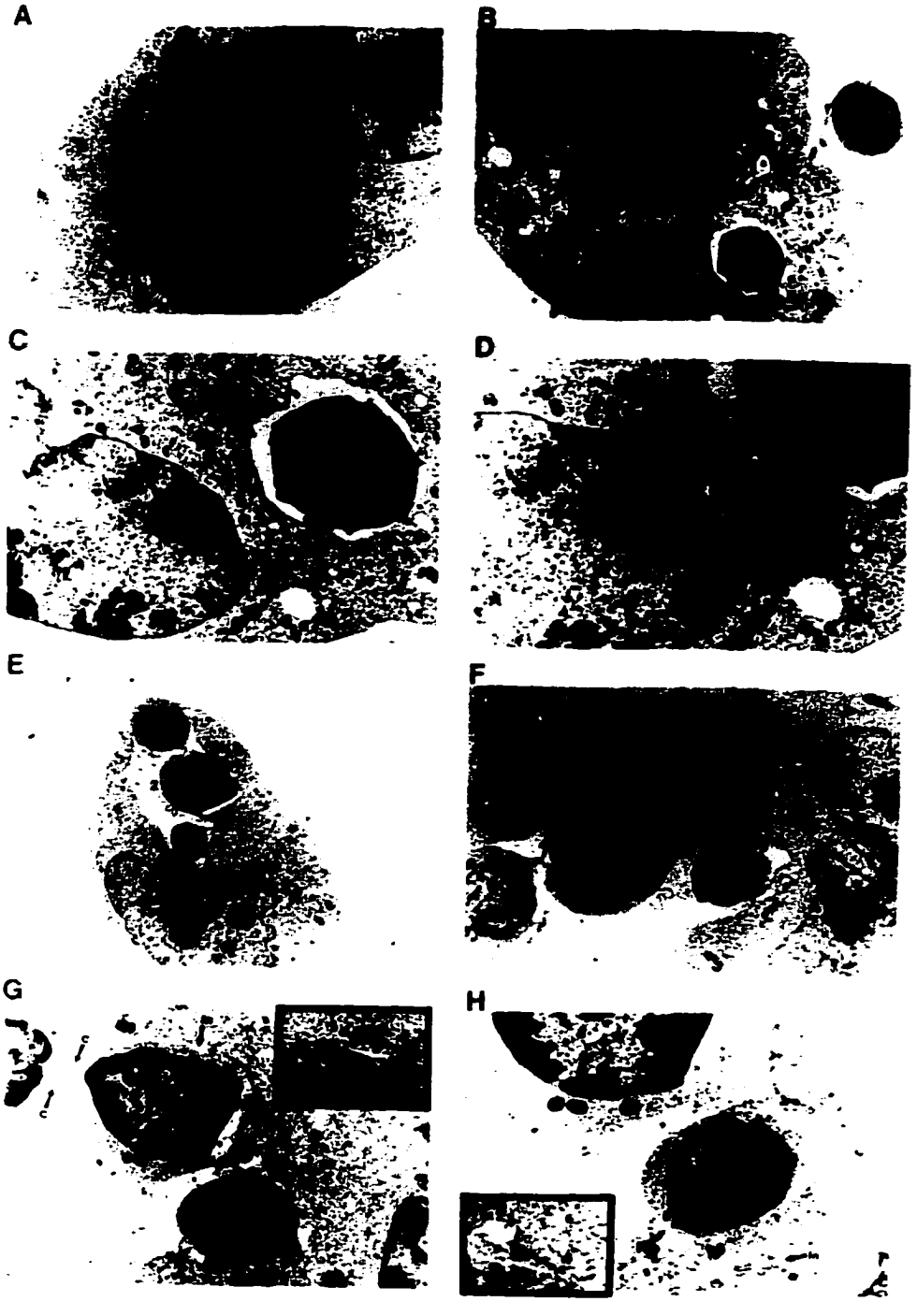


Fig. 4. Thymocyte Movement Within TNC Vacuoles. Time lapse video of a SVT-II2 TNC containing several bound and internalized thymocytes. The thymocytes labeled 1-5 are internalized. Internalized cells can be distinguished from bound cells because they are phase dark. Movement of these thymocytes is demonstrated by changes of the location of individual thymocytes relative to one another with increasing frame 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).

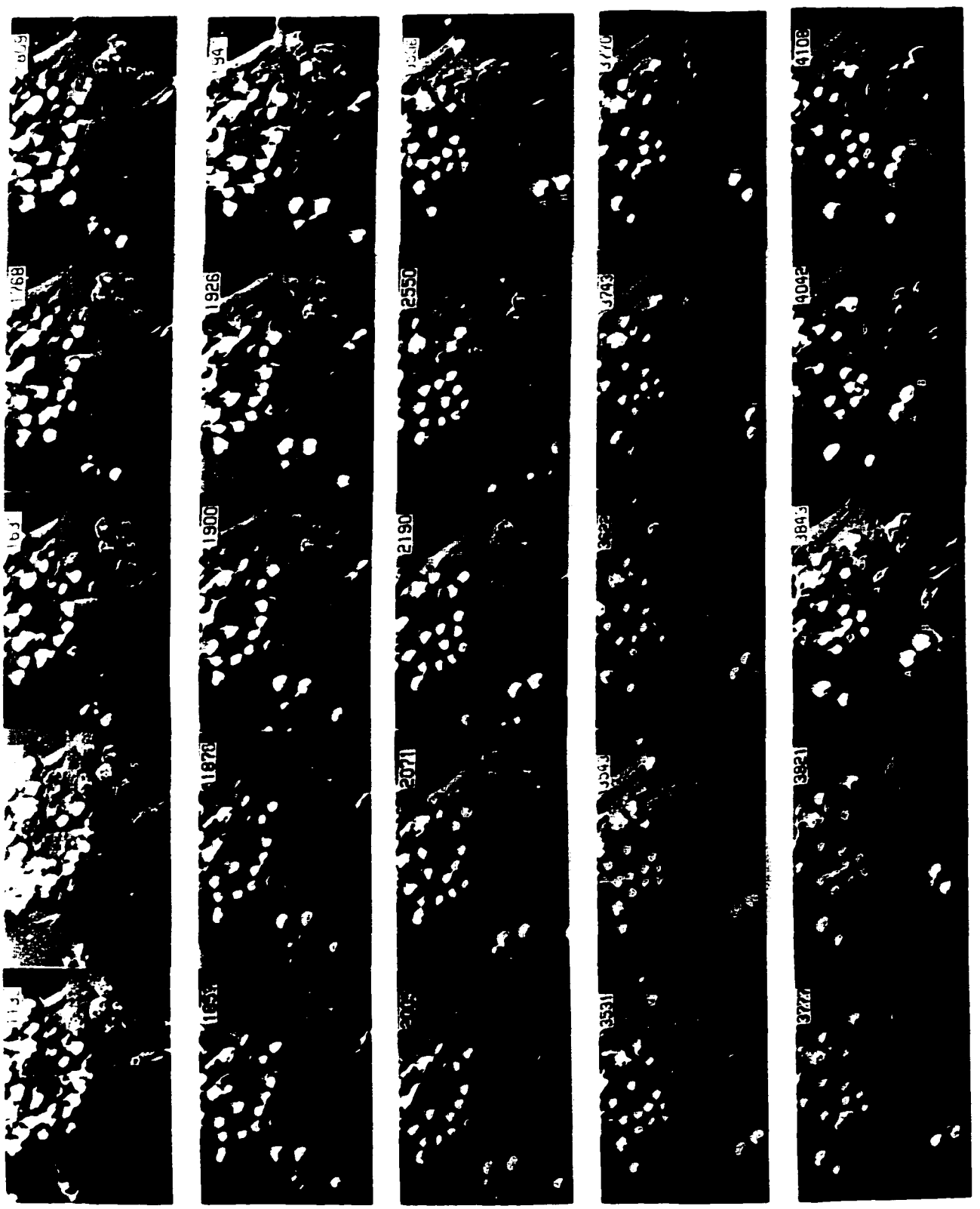


Fig. 5. Thymocyte Release. Thymocytes internalized into SVT-II2 cells were analyzed with long term video microscopy. The cell marked X is released in the series of micrographs. The cells labeled 1 and 2 are bound to the external surface of the TNC. The cell labeled X is shown to be completely separated from the TNC in frame 0744 (arrows).

Table 1. (A.) Phenotype. Freshly isolated thymocytes from 2-week- to 1-month-old mice were cocultivated with monolayers of SVT-II2. The thymocytes were allowed to bind and internalize. Unattached cells were removed by washing. The percentage of each internalized thymocyte subset was determined after staining with antibodies to both CD4 and CD8. (B.) Viability. Again, freshly isolated thymocytes were cocultivated with monolayers of SVT-II2. The thymocytes were allowed to bind and internalize. Unattached cells were removed by washing. The number and percentage of dead cells in both the bound and internalized populations were determined after staining with propidium iodide. As a control, freshly isolated thymocytes were centrifuged onto monolayers of SVT-II2 cells and unbound cells were removed with washing , as described above. The bound cells were then removed with vigorous washing and placed in culture for 48 hr without TNCs. At Time 0, 2.1% of the thymocites were P.I.+. A 12-hr time point was not done. At 24 hr 37.3% were P.I.+, and at 48 hr 69% were P.I.+. Of these cells, 96.2% were cellsurface CD4+CD8+ at Time 0. As another control, we sorted double-positive cells. These double-positive thymocytes were also incubated in growth media without SVT-II2 cells. The percentage death was as follows: Time0, 2.3%; 24 hr, 36.2%; 48 hr, 79.3%. P.I., propidium iodide.

TABLE 1

Phenotype and Viability of Internalized Thymocytes

Time (hr)	Number Internalized	No. CD4+CD8+ (%)	No. CD4-CD8- (%)	CD4+	CD8+
		A. Phenotype			
12	409	393 (96)	16 (4)	0	0
24	432	412 (95.4)	20 (4.6)	0	0
36	518	491 (94.8)	27 (5.2)	0	0
48	357	342 (95.7)	15 (4.3)	0	0

	Total no. cells	No. Internalized	No. internalized P.I. positive	No. bound	No. Bound P.I. positive
			B. Viability		
12	486	241	11 (4.6)	245	0
24	659	370	19 (5.1)	289	1 (.03)
36	583	326	31 (10.5)	257	1 (0.4)
48	106	78	3 (3.8)	28	0

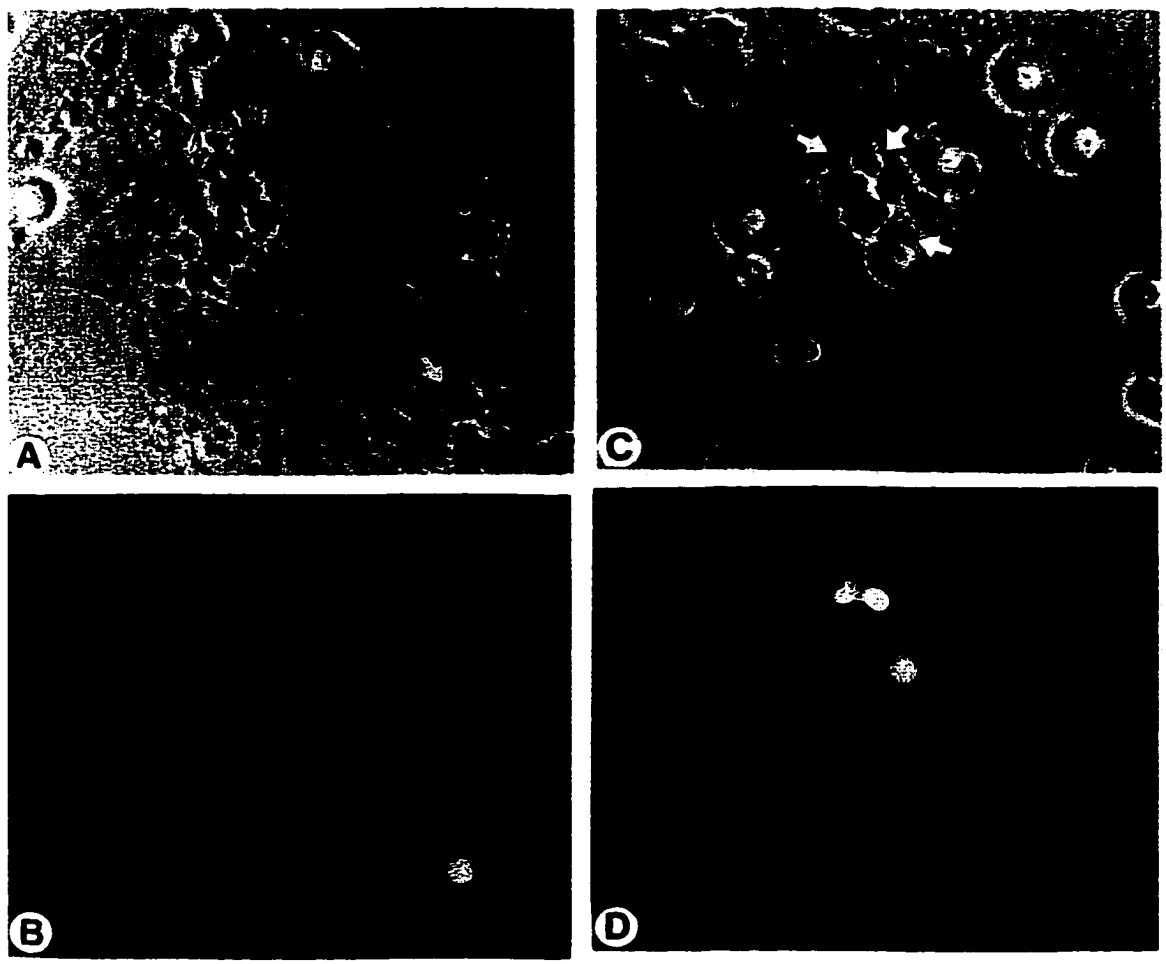
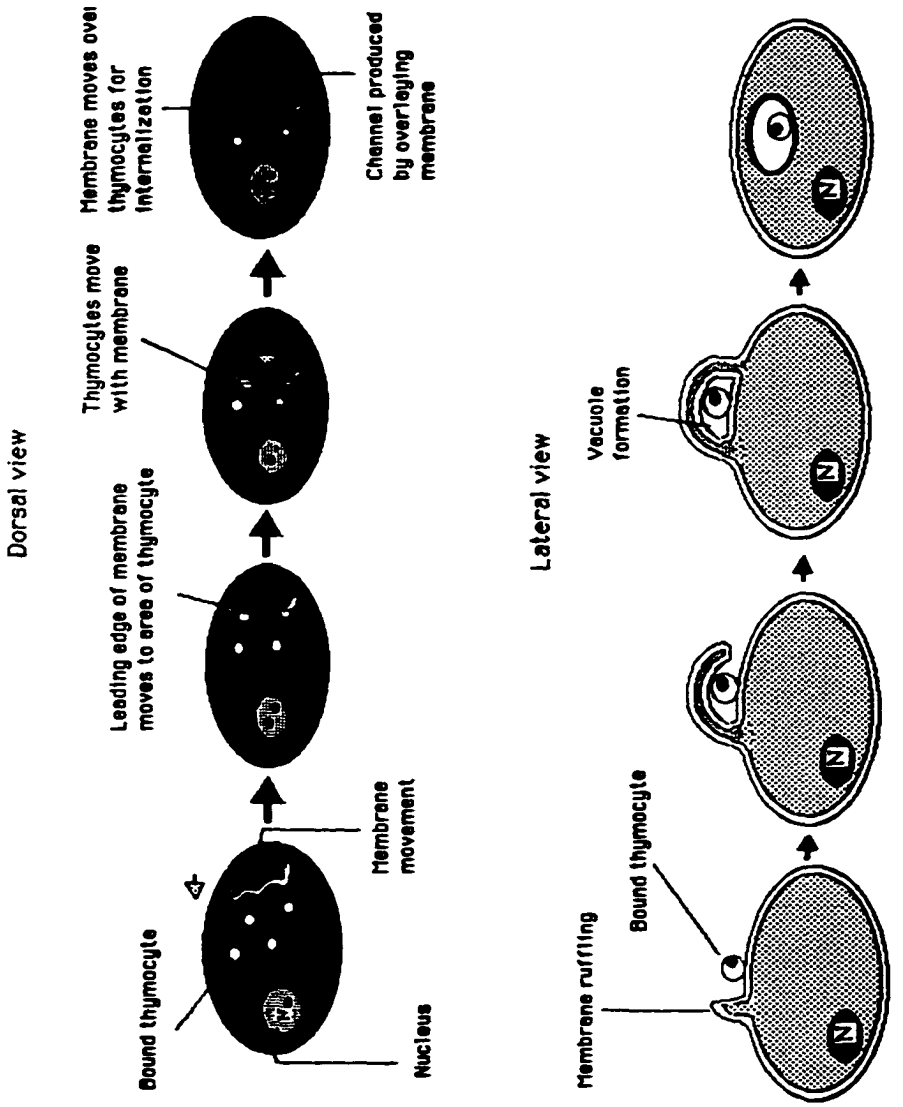


Fig. 6. Viability of Internalized Thymocytes. TNCs incubated with freshly isolated thymocytes were washed and exposed to propidium iodide (phase, A and C, flurescense, B and D, respectively). The nuclei of dead cells stain brightly (arrows). The nuclei of SVT-II2 TNCs, and those of viable internalized thymocytes are shown to be unstained.

Fig. 7. Model of Thymocyte Internalization by TNCs. Both the dorsal and lateral views show waves of membrane to develop after thymocyte binding. The wave of membrane moves to the area of the bound thymocytes. Bound thymocyte move with the wave along the external surface of the TNC. Internalization occurs when the TNC membrane moves over the top of bound thymocytes, and fuses to itself in an area on the other side of the trapped cell. Thymocyte movement inside of the TNC is proposed to occur within channels formed from the overlaying membrane.



CHAPTER 2

Title: **Thymic Nurse Cell Rescue of Early $CD4^+CD8^+$ Thymocytes From Apoptosis**

Authors: **Mark Pezzano, Yang Li, Deborah Philp, Coral Omene, Mary Cantey, Gregory Saunders, and Jerry C. Guyden**

Department of Biology, The City College of New York, 138th St at Convent Ave, New York, New York, 10031.

Abstract

We have now developed temperature sensitive lines of thymic nurse cells (TNCs), using the SV40 viral mutant tsA58, that maintain the ability to selectively internalize a subpopulation of αβ $TCR^+CD4^+CD8^+$ thymocytes *in vitro*. One line, 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 greatly diminished at 38°C, the temperature at which thymocyte binding was not observed. 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} $PNAr^{high}$ cells to the more mature TCR^{int} $PNAr^{low}$ phenotype but no changes in cell surface levels of HSA nor CD69 were detected. The

rescue activity of tsTNC-1 cells at 32°C was drastically reduced 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.

Key words

Thymic nurse cells, Apoptosis, Positive selection, Temperature sensitive SV40, Double positive thymocytes.

Introduction

Thymic nurse cells are unusual because they contain hundreds of thymocytes in specialized vacuoles completely enclosed within their cytoplasmic membrane (Wekerle and Ketelson, 1980; Wekerle et al., 1980; Andrew and Boyd, 1985). The function of this unique multicellular structure is unknown but several reports suggest that TNCs play a role in thymic education (De Waal Malefijt et al., 1986; Lorenz and Allen, 1989a and b). It has been difficult to study TNCs because only about 10^5 cells can be isolated per mouse thymus. Further, TNCs are fragile and many lyse during their isolation. These experimental limitations have prevented a conclusive identification of the TNC thymocyte population(s). It is

important to note that the TNC multicellular phenotype is the only characteristic used for their identification, which precludes experiments designed to study complex formation. Using TNC lines developed in our laboratory, we have been able to describe the internalization process (Philp et al., 1993) and to show that a subset of the αβ $TCR^+CD4^+CD8^+$ thymocyte population is exclusively bound by and internalized into TNCs (Li et al., 1992). These results suggest that the interaction between the two cell types is specific and may be important to the process of thymic education.

Here, we report the development of temperature sensitive lines of TNCs using tsA58, a temperature sensitive mutant of the SV40 virus (Lai et al., 1975). One cell line, tsTNC-1, was used to investigate the function of TNCs in T cell development. TsTNC-1 cells bind and internalize double positive thymocytes at 32°C, but at 38°C, binding was reduced to background levels and internalization was undetectable. From previous studies we discovered that TNC binding was restricted to viable thymocytes, while both live and dead cells were detectable within their specialized cytoplasmic vacuoles (Li et al., 1992). Also, single positive thymocytes were not found in association with TNCs. To determine TNC function during T cell development, we performed comparative studies of $CD4^+CD8^+$ thymocytes obtained after co-incubation with tsTNC-1 cells at 32°C versus those collected from cultures grown at 38°C. The results of these experiments show TNCs to have the capacity to rescue a selected double positive subset from programmed cell death, while inducing apoptosis in the remaining fraction. The rescued thymocyte population remained viable for over 4 days in cultures containing TNCs. Although TNCs bind double positives that showed immature expression levels of the TCR and the PNAr (TCR^{low} $PNAr^{high}$), the rescued subset expressed a

more mature TCR^{int} $PNAr^{low}$ phenotype. Rescued thymocytes also expressed high levels of cell surface CD4 and CD8 but cell surface levels of both HSA and CD69 (Bendelac et al., 1992; Swain et al., 1988; Nikolic Zugic et al., 1990) remained unchanged. The rescue activity of tsTNC-1 cells was not observed at 38°C, the temperature at which binding and internalization were undetectable. Rescue was greatly diminished in co-incubation experiments at 32°C in the presence of antibodies against either class I or class II MHC proteins. Our results suggest that TNCs play a role in the rescue of double positive thymocytes during selection, and the survival of selected $CD4^+CD8^+$ thymocytes requires binding to TNCs and an interaction between the TCR and the MHC complex. TNC-rescued thymocytes mature from TCR^{low} $PNAr^{high}$ $CD4^{low}CD8^{low}$ to TCR^{int} $PNAr^{low}$ $CD4^{high}CD8^{high}$ expressing cells, a stage within the double positive window that precedes HSA and CD69 expression level changes.

Materials and Methods

Isolation of Thymic Nurse Cells

Thymic nurse cells were isolated using fetal calf serum density gradients as described by Werkele (1980). TNCs were allowed to release enclosed thymocytes and to form monolayers before exposure to tsA58 (originally obtained from Dr. P. Tegtmeyer) at a multiplicity of 10 PFU/cell at approximately 5 X 10^4 cells/ml in RPMI plus 2 μg/ml polybrene. After an adsorption period of 30 minutes, the cells were diluted with growth media and maintained in culture at 32°C before cloning using limiting dilution.

Binding Assay

The binding assay was previously described in Pezzano et al. (1991). Briefly, 2 X 10^6 thymocytes were added to a monolayer of 1 X 10^4 TNCs. After an overnight incubation period, unattached lymphocytes were removed by three washes with PBS. All of the thymocytes collected in the wash were pooled and counted. The percentage of bound cells was determined as a function of the difference between the total number of cells added and the number of unattached cells divided by the total number of cells added times 100.

Growth Curve

TsTNC-1 cells were seeded in 35-mm dishes at a density of 1 X 10^4 cells/plate. Multiple plates were prepared. Half of the cultures were incubated at 32°C and the other half were placed at 38°C. Cell counts were performed at 24 hour intervals using a hemacytometer. Viability was determined by Trypan blue exclusion. Each point represents the average cell count of three plates.

Coculture 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 preparation for FACS sorting. Thymocytes were resuspended at 5 X 10^6 cells/ml and CD4 CD8 double positive thymocytes were sorted (> 97 % pure) using a Colter Epics Elite (model F09102). 5 X 10^6 sorted thymocytes were incubated with 2 X 10^5 tsTNC-1 cells, or 3T3 cells for either 48 or 96

hours 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.

Scanning Electron Microscopy

Monolayers of tsTNC-1 cells were grown to an appropriate density at 32°C in tissue culture dishes. Half of the dishes were then shifted to 38°C and incubated for an additional 48 hours. The cells were then washed with RPMI and fixed at 4°C for one hour in 2% gluteraldehyde made in 1 X PBS. The samples were washed twice in 1 X PBS prior to a 30 minute incubation in 1% osmium tetroxide. Each plate was washed 3 times with 1 X PBS and dehydrated in ascending percentages of ethyl alcohol. The samples were treated three times with 100% ethyl alcohol followed by critical point drying using a CPD 030 critical point dryer. The samples were mounted on stubs using silver paint and dried overnight at 60°C. The stubs were then sputter coated with gold at a thickness of 10 nm. A Zeiss Scanning Electron Microscope was used to view samples. Photographs were taken using Kodak 4415 film.

DNA Fragmentation Assay

Thymocytes (3 X 10^6) 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/ml of

proteinase K) and incubated for 1.5 hr at 50°C. The samples were extracted three times with phenol-chloroform and precipitated with ethanol. An equal concentration of each sample was resuspended in TE after drying and loaded onto 2% agarose gels containing 0.05 mg/ml ethidium bromide (Sentman, et al. 1991). Apoptosis was induced in control cultures by exposing freshly isolated thymocytes to 10 mM hydrogen peroxide for 2 hours before the required incubation period. Photographs were taken under UV light using Polaroid film.

Northern Blot Analysis

Total cellular RNA was isolated from tsTNC-1 cells with 4 M guanidine thiocyanate and centrifuged in a CsCl gradient. MRNA was isolated using oligo-dT columns. 10 µg of whole cell RNA was subjected to electrophoresis on a 1% agarose gel containing formaldehyde in 0.2 M morpholinopropanesulfonic acid-acetate and 10 mM EDTA for 560 volt hours. After transfer to nitrocellulose, each blot was hybridized using a 2.7Kb BamH I to Bgl I fragment of the SV40 genome that spans the early region genes as a probe for large T antigen expression.

Results

Development and Characterization of Temperature Sensitive Thymic Nurse Cell Lines

Fresh thymic nurse cells were isolated from C57BL/6 mice and purified on fetal calf serum density gradients. The resulting cells were allowed 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 (Figure 1A) and had a higher growth rate (Figure 1C) 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 (Figure 1B). Loss of the transformed phenotype upon the shift to 38°C is concurrent with the loss of SV40 early region gene expression which contains the large T antigen sequence (Figure 1D). After about 48 hours at 38°C, mRNA for large 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 (Geenen et al., 1988), a neuroendocrine cell marker which has been shown to be expressed exclusively by TNCs in the thymus (Table 1). TsTNC-1 cells also expressed the cortical marker ER-TR-4, but were negative for ER-TR-5, an antibody that recognizes a thymic medullary antigen (van Vliet et al., 1985).

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 (Li et al., 1992).

Binding assays were performed using tsTNC-1 cells at both 32°C and 38°C. TsTNC-1 cells maintained the ability to bind and internalize fresh thymocytes at 32°C, but not at 38°C (Figure 2A). The wild type SV40-transformed TNC line MP5 showed equivalent binding efficiencies at both temperatures. A thymocyte internalized into a specialized TNC vacuole at 32°C is shown in Figure 2B.

TsTNC-1 Rescue of Double Positive Thymocytes from Apoptosis

To determine the effect of TNCs on thymocyte development *in vitro*, co-incubation experiments were performed using FACS-sorted double positive thymocytes. Sorted double positive thymocytes, at a purity of over 97%, were placed on monolayers of tsTNC-1 cells at either 32°C or 38°C, and reanalyzed after 48 (Figure 3A) or 96 hours (Figure 3B) using forward scatter. The ratio of viable to nonviable thymocytes detected in tsTNC-1 cultures grown at 32°C (74 % viability) was much higher than that observed in tsTNC-1 cultures at 38°C (22 % viability) or 3T3 cultures (54 % viability) after a 48 hour co-incubation period. By day 4, survivors were sparse in control samples (1.9 % viability of thymocytes with 3T3; 0.5 % viability with tsTNC-1 at 38°C) but the ratio of live to dead thymocytes remained relatively high in samples grown at 32°C in the presence of tsTNC-1 cells (55 % viability). Analyses of the CD4/CD8 phenotype of thymocytes obtained from day 4 cultures revealed that the nonviable thymocytes produced a reduced level of CD4 and CD8 staining under all incubation conditions (Figure 3, third histogram in each set, open gates). These data are consistent with those published by Swat et al. (1991) which showed double positive thymocytes with very low expression levels of both CD4 and CD8 to be apoptotic. Viable double positives in tsTNC-1

cultures at 32°C expressed higher levels of both CD4 and CD8 than those detected in control samples (Figure 3C, panels 1 and 2). The expression level of both CD4 and CD8 on rescued thymocytes was higher than the majority of double positives obtained in freshly isolated samples. The subset of double positives that express CD4 and CD8 levels equivalent to that found on TNC-rescued thymocytes represents 20.5 % of the total population in freshly isolated samples (Figure 3C, panel 3).

Upon close examination, it appears that cultures containing tsTNC-1 cells at 32°C produce an increased number of both CD4 and CD8 single positives. If this represented an actual shift from double positives to single positives, the ratio of viable double positives to single positives within this closed system would decrease over time in comparison to control cultures. However, a comparison of the percentage of viable thymocytes within each phenotype quadrant reveals that the ratio of double positives to single positives in tsTNC-1 cultures at 32°C remains relatively unchanged from 48 to 96 hours (Figure 3A and B). By day 4, the ratio of double positives to single positives decreased drastically in control cultures as a result of death within the double positive population. We interpret these data to mean that the appearance of single positives in tsTNC-1 cultures at 32°C resulted from a fixed number of sorted double positive thymocytes that were preselected to shift *in vivo*. The size of the single positive thymocyte population reflects the longevity of all rescued thymocytes and not an *in vitro* shift from double to single positives. Further, single positive thymocytes are not found within the TNC complex (Li et al., 1992).

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 (Li et al., 1992). Apoptotic 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 (Wyllie, 1980). The DNA fragmentation assay was exploited for this investigation (Figure 4). Viable versus nonviable thymocytes were sorted using forward scatter after co-incubation with TNCs and 3T3 cells. It is important to note that this experiment was designed to analyze TNC-interactive thymocytes exclusively. The thymocytes analyzed were all TNC-adherent because nonadherent double positives were removed after a short incubation period (2 hours). The resulting thymocytes were collected and sorted after 4 days into viable versus nonviable subsets using forward scatter (see gates in 4 day forward scatter histogram for tsTNC-1 at 32°C in Figure 3). Fragmentation was undetectable in thymocytes sorted under the viable gates after a 96 hour coculture with tsTNC-1 cells at 32°C (Figure 4A, lane 4). Several attempts were made to recover DNA from the nonviable population (Figure 4A, lane 5) and as many as 6 X 10^6 events were collected and analyzed but resolvable concentrations were never obtained. Upon visual examination, a large percentage of the cells (sorted under the nonviable gates) was completely destroyed. We interpret these results to mean that both live and dead cells result from the TNC-adherent population, and many of the thymocytes, termed nonviable, disintegrate with time in culture due to the process of apoptosis. The live subset exist as a function of a reduced level of apoptosis. We then examined cocultured thymocytes separated into bound versus unbound fractions. Unlike the experiment reported in Figure 4A, which only examined the

TNC-interactive 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 Figure 3, and the thymocytes resulting from 1 and 2 day cultures were analyzed using the DNA fragmentation assay. Figure 4B shows DNA fragmentation to be greatly reduced in the TNC-bound fraction at 32°C when compared to unbound populations found in the same cultures at 32°C, in tsTNC-1 cultures grown at 38°C, or in 3T3 cultures. Collectively, these data suggest that the 4 day viable thymocyte subset results from the TNC-bound population, however, both viable and apoptotic cells exist within the TNC-interactive population.

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 (McCarthy et al., 1988), 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 αβ TCR^{low} double positive population (Figure 5A). At 48 hours, a shift to the αβ TCR^{int} phenotype was detected in the rescued population of tsTNC-1 cultures at 32°C (Figure 5A and C). Again, the percentage of surviving thymocytes was much higher in TNC cultures than that detected in control cultures (Figure 5A).

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 (Ohashi et al., 1990). 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 (Li et al., 1992). These framework antibody preparations were used to determine their effect on rescue. As seen in Figure 6, the percent rescue was reduced in tsTNC-1 cultures at 32°C containing antibodies against either determinant (Figure 6C and D), 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 (Chan et al., 1993; Wagner et al., 1980 a and b), 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 7A). 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 7B). These data are consistent with reports that show the shift to the more mature HSA^{low} phenotype to occur only after thymocytes become single positives (Bendelac et al., 1992). In that same study, CD69 was shown to be initially expressed on a small (3 %) subset of $TCR^{low/int}$ double positive thymocytes, while maximal expression was detected on TCR^{high} double positives. It appears that the thymocyte interaction with TNCs causes a shift to a stage within the double positive phase that precedes CD69 expression. To summarize the results described above, the phenotype of the thymocyte subset that binds TNCs is $\alpha\beta$ $TCR^{low}CD4^{+}CD8^{+}PNAr^{high}$,

while the rescued population released from this interaction is αβ $TCR^{int}CD4^{high}CD8^{high}PNAr^{low}$ thymocytes.

Discussion

The process of restriction during T cell development has been reported to involve the attachment of thymocytes to stromal cells of the cortex (Fowlkes, et al. 1989). Several studies report thymocyte selection during restriction to be facilitated through an interaction between the TCR on the surface of developing thymocytes and MHC proteins presented on cortical cells (Sprent, 1978; Marrack et al., 1988; Zuniga-Pflucker et al., 1989; Sha et al., 1988; Teh et al., 1988; Berg et al., 1989; Kaye et al., 1989). Although the thymocytes participating in this interaction have been examined extensively, the characterization of their stromal counterparts is preliminary at best. Thymic nurse cells are unique stromal cells found in a multicellular complex with double positive thymocytes in the subcapsular region of the thymic cortex (Wekerle and Ketelson., 1980; Wekerle et al., 1980; Andrew et al., 1985). TNCs are unusual because they are MHC class II-expressing epithelial cells, and have the capacity to internalize αβ $TCR^{+}CD4^{+}CD8^{+}$ thymocytes into specialized cytoplasmic vacuoles (Li et al., 1992). Unique contact structures have been shown to exist between the membranes of enclosed thymocytes and the cytoplasmic vacuoles (Philp et al., 1993). Although the function of this interaction remains unknown, these data suggest a role for TNCs in the process of thymic education.

The results of our earlier report showed that TNCs to bind 20 % of the double positive population (Li et al., 1992). That study also revealed that

binding and internalization were restricted to live cells, but 11% of captured thymocytes die within TNC cytoplasmic vacuoles (Philp et al., 1993). When double positive thymocytes were examined in cultures not containing TNCs, 80% of the cells die within 48 hours. These results suggest that thymocyte death is reduced as a function of the TNC interaction. In this studies reported here, we developed temperature sensitive lines to verify that contact between the TNC and its interactive double positive subset is required for survival during this stage of development. At 38°C, tsTNC-1 cells do not bind thymocytes and 99.5% of the thymocytes die after 4 days in culture (Fig. 3). On the other hand, apoptosis was shown to be reduced in the TNC- interactive thymocyte population at 32°C. TNC rescue was consistently detected in all of the assays reported in this study (See Figures 3, 5, 6 and 7). 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 (Fig. 3B). 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 of apoptosis. These data are consistent with studies that report positive and negative selection to occur within the same thymic stromal epithelial cell type (Hugo et al., 1994). 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 (Spain and Berg, 1992).

The characterization of the TNC-bound double positive subset shows binding to be restricted to the immature TCR^{low} $PNAr^{high}$.

expressing phenotype (Fig. 6 ,7). The viable thymocytes released from this interaction maintained their double positive phenotype but expressed cell surface TCR and PNAr levels indicative of a shift to a more mature phenotype within the double positive stage. Previous reports showed double positives to be homogeneously high expressors of the PNAr, and the switch to the $PNAr^{low}$ phenotype to occur upon transition to the single positive phenotype (Bendelac et al., 1992). Our data show a decrease in the PNAr expression level to occur within the double positive window. Also, rescued thymocytes expressed high levels of cell surface CD4 and CD8 (Fig. 3). High level expression of both CD4 and CD8 can be interpreted two ways. Either the interaction with TNCs induces high level expression of CD4 and CD8, or thymocytes that express high levels of CD4 and CD8 are selectively rescued. The results of the experiment reported in Figure 3 show the initial TNC-interactive double positive subset to express lower levels of both CD4 and CD8 when compared to the rescued population. These data support the idea that the thymocyte/TNC interaction results in a shift of the rescued subset from $CD4^{low}CD8^{low}$ cells to the $CD4^{high}CD8^{high}$ phenotype. In either case, rescue was shown to be associated with a shift to the TCR^{int} $PNAr^{low}$ phenotype along with high level expression of both CD4 and CD8. The thymocyte interaction with TNCs appears not to provide the stimuli required for the shift to the single positive stage of development concomitant with high TCR expression levels, however, the switch to the TCR^{int} phenotype in the rescued population is indicative of positive selection (Ohashi et al., 1990). Upon closer examination, it appears that cultures containing tsTNC-1 cells at 32oC produce an increased number of both CD4 and CD8 single positives. If this represents an actual shift from double positives to single positives,

the ratio of viable double positives to single positives within this closed system would decrease over time in comparison with that of control cultures. However, a comparison of the percentage of viable thymocytes within each phenotype quadrant reveals that the ratio of double positives in tsTNC-1 cultures at 32°C remains relatively unchanged from 48 to 96 hrs. (Figs 3A,3B). By day 4, the ratio of double positives to single positives decreased drastically in control cultures as a result of death within the double positive population. We interpret these data to mean that the appearance of single positives in tsTNC-1 cultures at 32oC resulted from a fixed number of sorted double positive thymocytes that were preselected to shift *in vivo*. The size of the single positive thymocyte population reflects the longevity of all rescued thymocytes and not an *in vitro* shift from double to single positives. Further, single positive thymocytes are not found within the TNC complex (Li et al., 1992).

Positive selection involves the TCR/MHC interaction (Berg et al., 1989; Marrack et al., 1989; Sha et al, 1988; Sprent, 1978; The et al, 1988; Zuniga-Pflucker et al., 1989). We showed previously that antibodies to MHC class I or class II antigens did not interfere with the TNC binding activity (Li et al, 1992). These same antibody preparations were shown to inhibit the TNC rescue activity (Fig. 4). We believe these results to mean that na interaction between the TCR and the MHC is required for TNC rescue activity but not for the binding of thymocytes to thymic nurse cells. Such a model has been proposed for other cortical epithelial cells that require integrins for binding of double positive thymocytes to facilitate the TCR/MHC interaction (Salomon et al., 1994).

In conclusion, our *in vitro* model system has been used to show that thymocytes binding to TNCs is required for rescue from programmed cell

death. Rescue involves an interaction between the TCR on developing thymocytes and the MHC antigens on TNCs. We also show that within the TNC-interactive population, there are both viable and apoptotic thymocytes. Finaliy, a subset of the surviving thymocytes matures within the double positive window of development.

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FIGURES AND LEGENDS

Figure 1. 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.

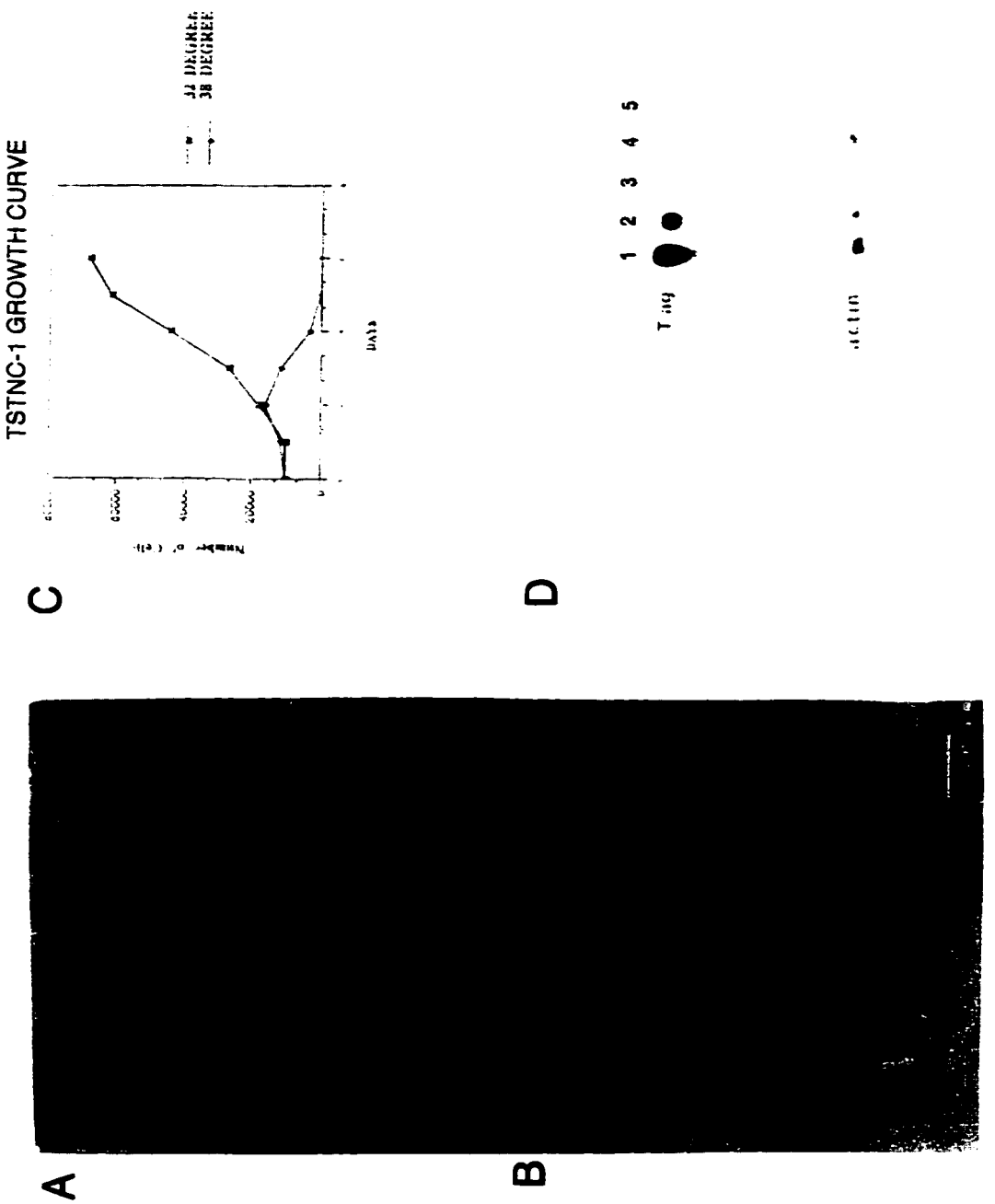


Fig. 2. Binding Assay

(A) 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.

(B) A transmission electron micrograph of a freshly isolated thymocyte internalized into the specialized cytoplasmic vacuole of a thymic nurse cell after an overnight incubation at 32°C. Magnification: top micrograph 10,000 X, bottom micrograph 20,000 X.

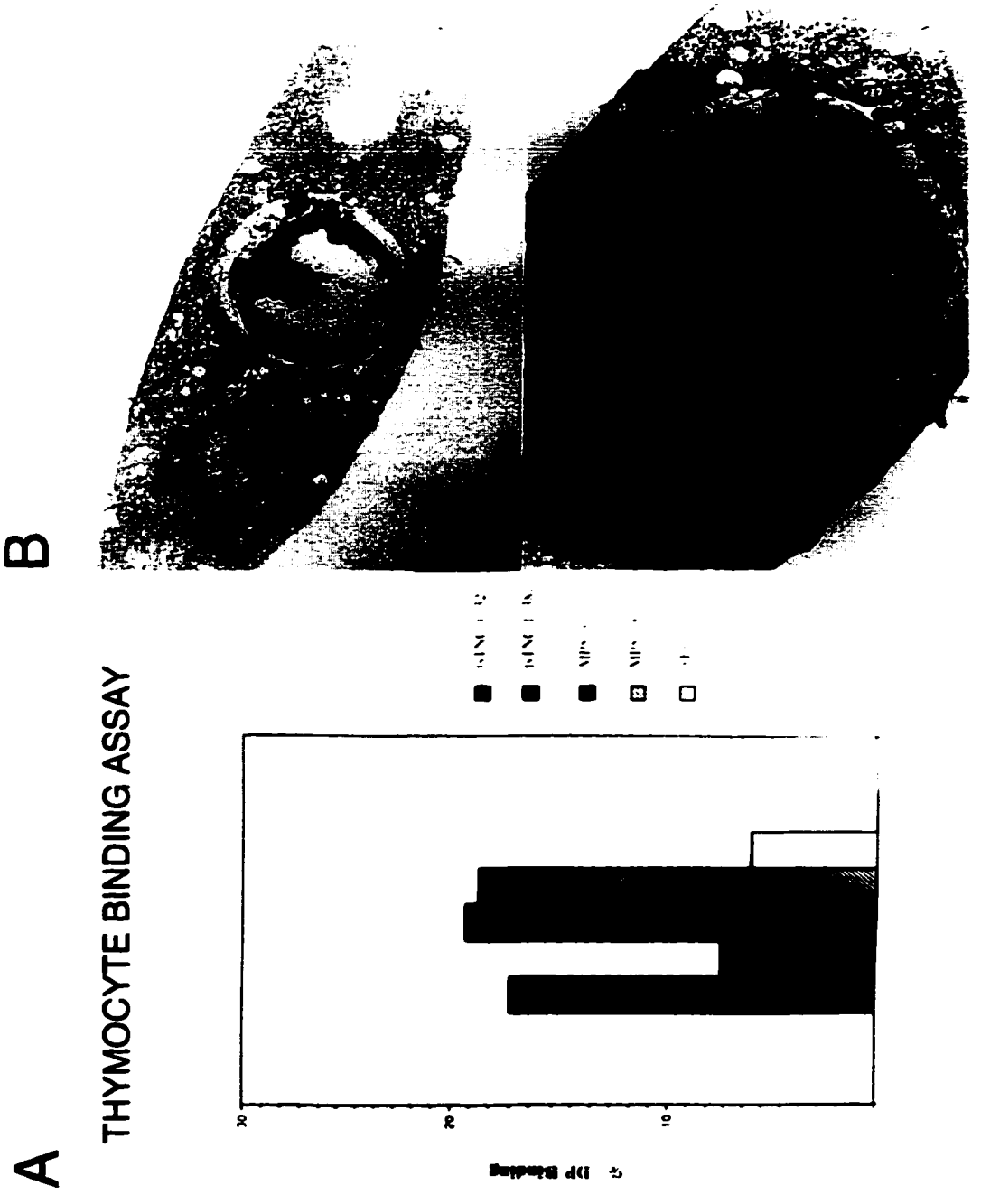
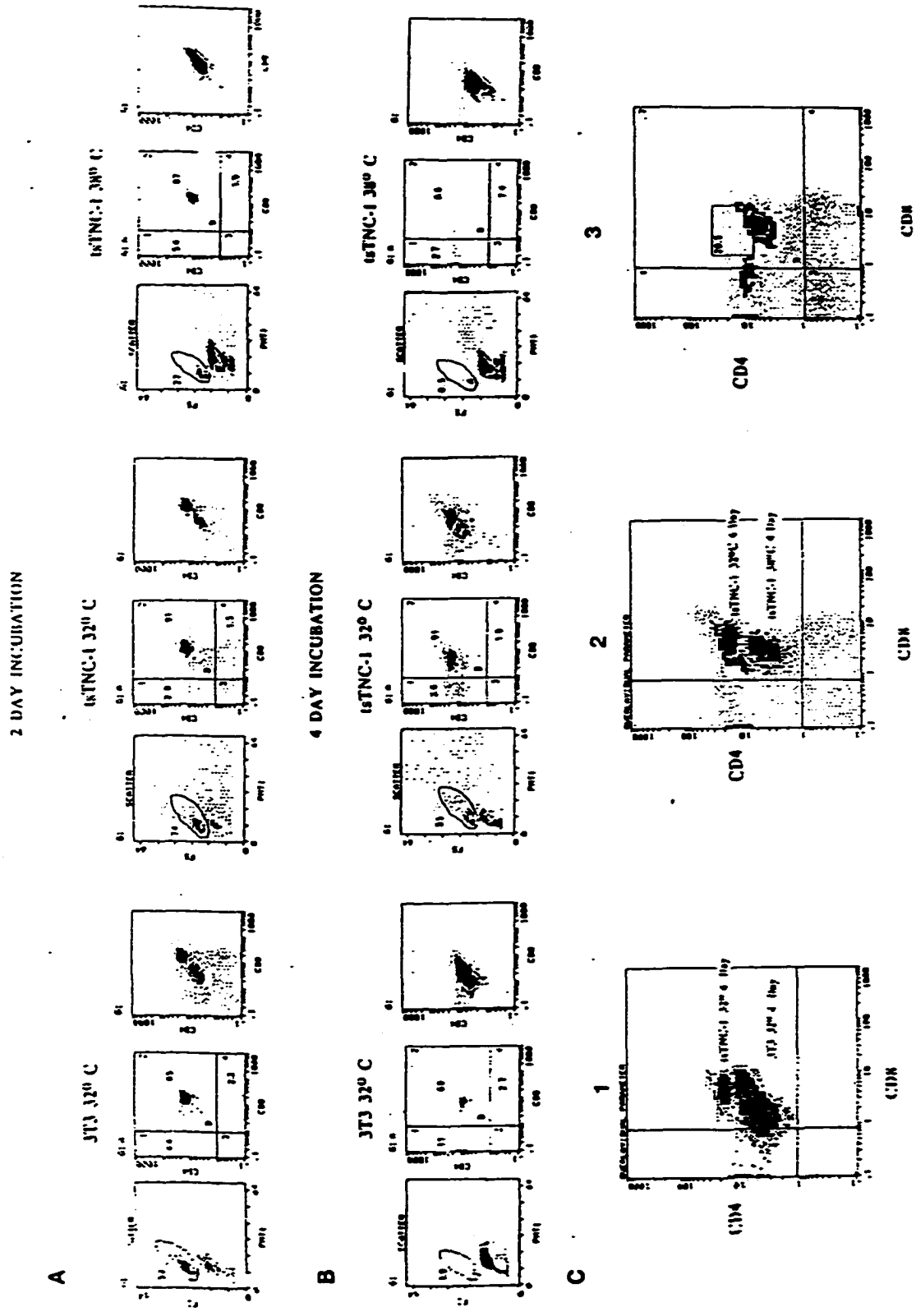


Fig. 3. Co-incubation Experiments

Freshly isolated thymocytes were stained with antibodies against CD4 and CD8 and sorted to a purity of over 97%. 5 X 10^6 sorted thymocytes were incubated with 2 X 10^5 tsTNC-1 at 32°C or 38°C, or with 2 X 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.



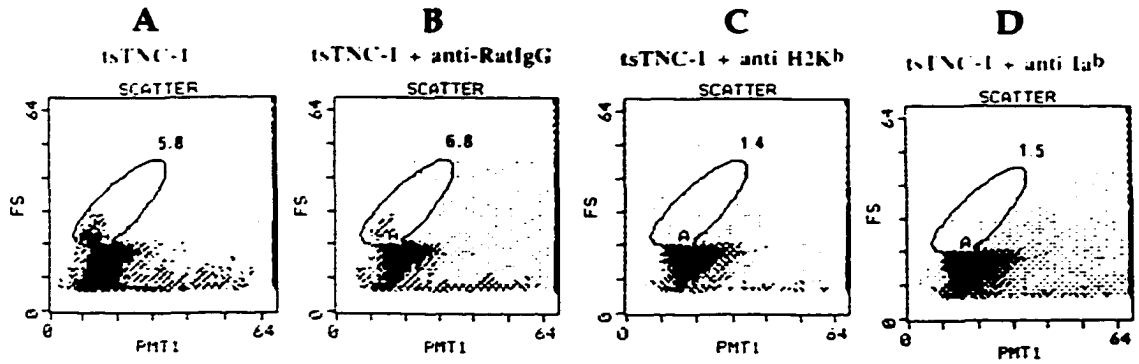


Fig. 4 *The effect of class I or class II MHC antibodies on thymocyte rescue.* Thymocytes were resuspended at 5 x 10^6 cells/ml and CD4 CD8 double positive thymocytes were sorted using a FACS Colter (model Elite) as described above. Sorted thymocytes (5 x 10^6) were incubated with 2 x 10^5 tsTNC-1. Co-cultures were maintained for 48 hrs. 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.

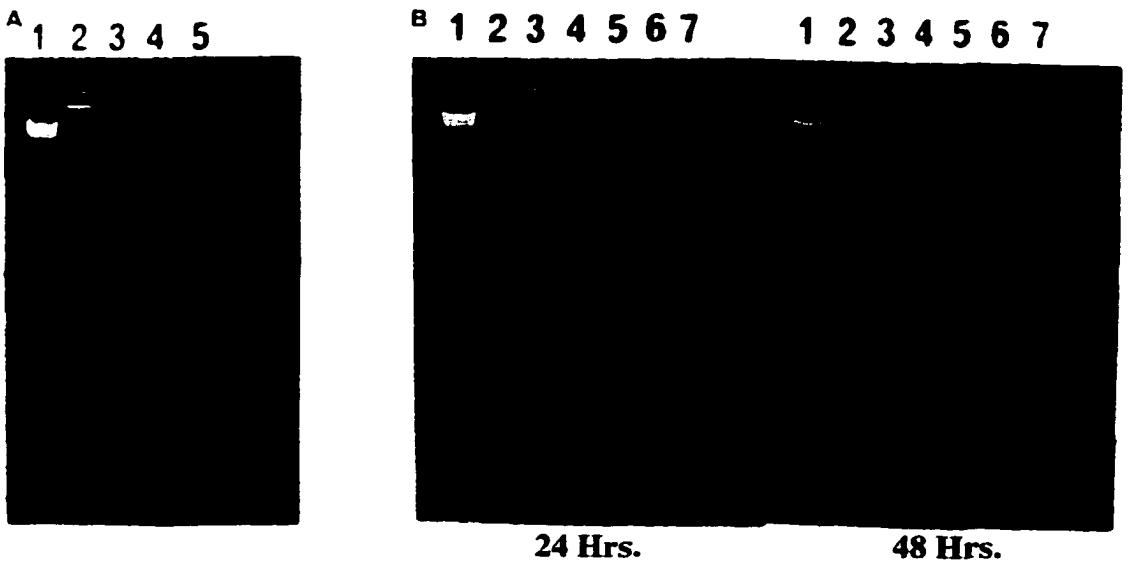


Fig. 5 *DNA fragmentation assay.* **A)** DNA recovered from TNC-adherent thymocytes after co-incubation for 4 days with tsTNC-1 cells at 32°C. Thymocytes were FACS-sorted into viable and non-viable populations before analysis using the DNA fragmentation assay. Lane 2: freshly isolated thymocytes treated for 2 hrs. 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: non-viable 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 hrs. 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 hrs. 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.

Fig. 6. Cell surface 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.

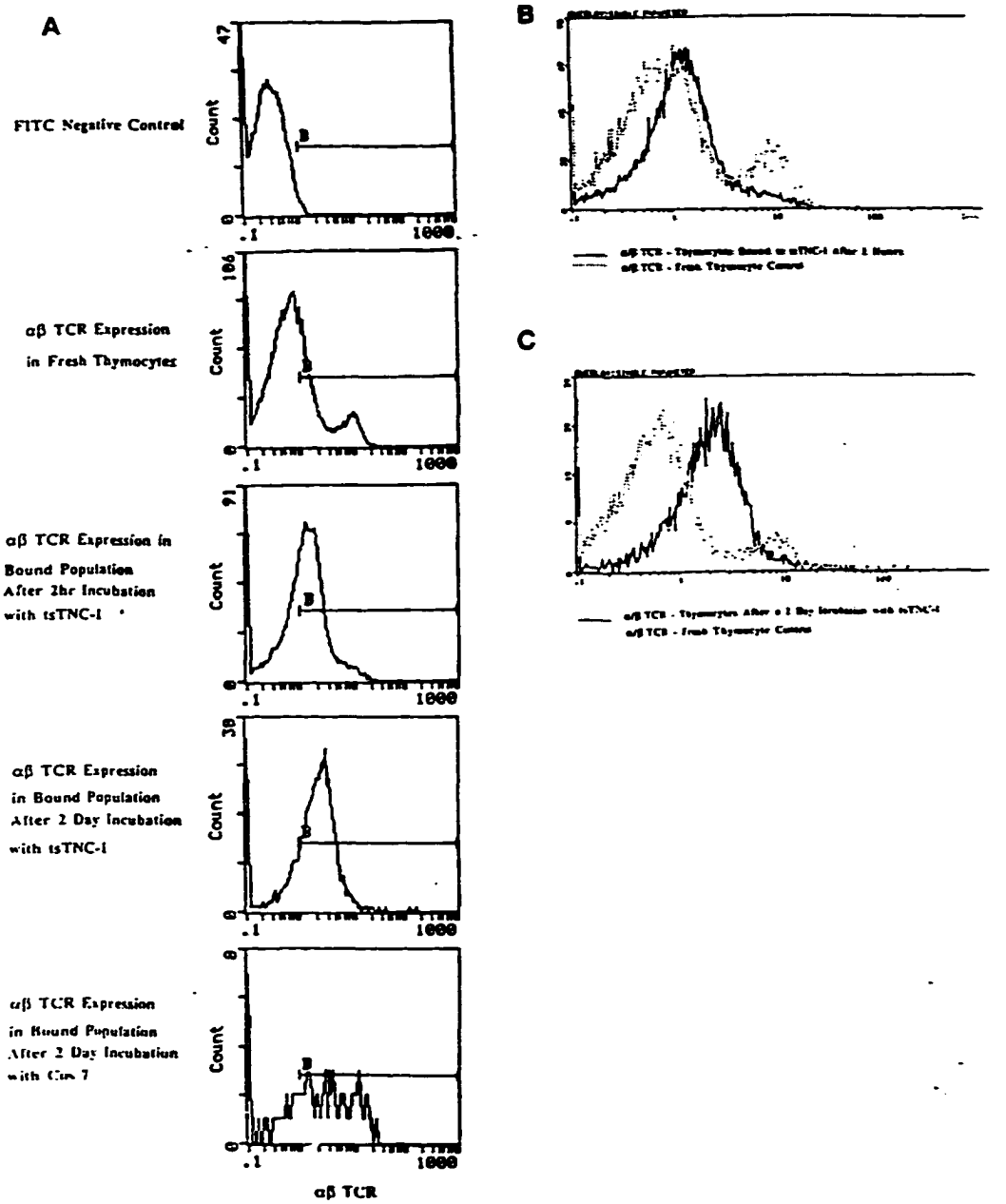
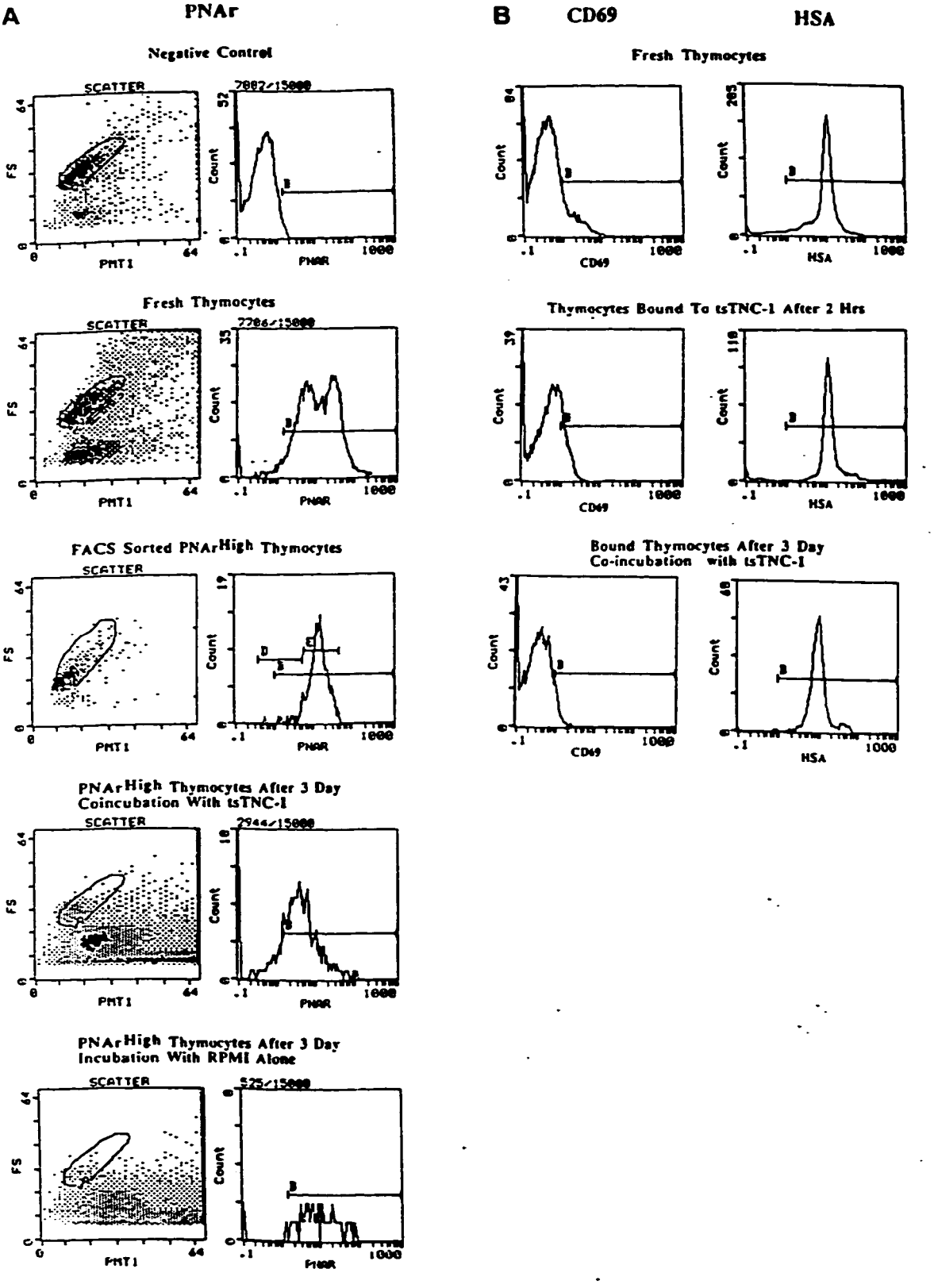


Fig. 7. Cell Surface Characteristics of Rescued Thymocytes
(A) $PNAr^{high}$ thymocytes were sorted using a FACS. 5 X 10^6 sorted thymocytes were incubated with 2 X 10^5 tsTNC-1 and allowed to bind. Unbound cells were removed by washing. The remaining thymocytes were recovered and analyzed for initial 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.



CHAPTER 3

Title: **Positive Selection by Thymic Nurse Cells Requires IL-1β and is Associated with an Increased Bcl-2 Expression**

Authors: **Mark Pezzano, Deborah Philp, Sharon Stephenson, Yang Li, Vincent Reid, Robert Maitta and Jerry C. Guyden**

Department of Biology, The City College of New York, 138th St. at Convent Ave., New York, New York, 10031.

Running Title: TNC Rescue of $CD4^+CD8^+$ Cells Involves IL-1β

Reprint request should be addressed to Dr. Jerry C. Guyden, The Department of Biology, The City College of New York, 138th St. at Convent Ave., New York, New York 10031.

Abstract

A temperature sensitive line of thymic nurse cells (tsTNC-1) that maintains the ability to selectively internalize immature $\alpha\beta TCR^{lo}CD4^+CD8^+$ thymocytes *in vitro* was used in long term co-incubation experiments to determine nurse cell function during the process of MHC restriction. The thymocyte subset released from its association with TNCs contained both viable and apoptotic

cells. The cells that remained within intra-cytoplasmic vacuoles died through the process of programmed cell death. Surviving or rescued thymocytes in the released population displayed an increase in Bcl-2 protein expression. The rescue activity of TNCs was drastically reduced with the addition of antibodies against either class I or class II MHC antigens to cocultures. A subset of the TNC-rescued population matured from the $\alpha\beta TCR^{lo}CD69^{-}$ phenotype to $\alpha\beta TCR^{hi}CD69^{+}$-expressing cells only when IL-1β was added to cocultures. These results suggest that TNC rescue of early double positive thymocytes from apoptosis is associated with an interaction between the TCR and the MHC and the onset of Bcl-2 expression. Maturation of thymocytes within the TNC-rescued population requires the costimulatory effects of IL-1β.

INTRODUCTION

Thymic nurse cells are unusual because they contain up to two hundred thymocytes in specialized vacuoles completely enclosed within their cytoplasmic membrane (1, 2, 3). The function of this unique multicellular structure is unknown but several reports suggest that TNCs have a role in the MHC restriction process during thymocyte development (4, 5, 6). Studies of TNC function have been problematic because only about 10^5 cells can be isolated per mouse thymus and many TNCs lyse during their isolation, making a conclusive identification of the TNC thymocyte population(s) difficult. Currently, TNCs can only be identified by their multicellular structure, which precludes experiments designed to study complex formation. TNCs are MHC class I and II positive cortical epithelial cells that have been shown to have antigen presenting capabilities (6, 7, 8). Using TNC lines

developed in our laboratory, we previously described the internalization process and have shown a subset of $\alpha\beta TCR^{lo}CD4^{+}CD8^{+}$ thymocytes to be the interactive population (9, 10). Long term co-incubation experiments in our laboratory revealed that TNCs produce viable and apoptotic thymocyte subsets. These data were consistent with studies that reported the potential for both positive and negative selection to occur within the same thymic stromal epithelial cell type (11). It is important to note that maturation within the surviving TNC thymocyte population has yet to be demonstrated.

The presentation of self molecules by MHC proteins expressed on specialized antigen presenting cells within the thymus is essential for thymocyte maturation as well as the deletion of self reactive clones. The mechanism of antigen presentation by thymic nurse cells was initially shown to be similar to that of thymic macrophages and medullary dendritic cells (5). A subsequent report showed TNCs to require the lymphokine IL-1β to facilitate effective antigen presentation (6). Antigen presenting cells transmit their developmental signals via an interaction with the αβTCR expressed on the cell surface of developing thymocytes (12, 13). It has been proposed that the exclusive engagement of the αβTCR produces a signal for clonal inactivation, but additional input from co-stimulatory molecules may alter the signal and result in clonal activation (6, 14, 15). The experiments that demonstrated the IL-1β requirement for complete TNC function were performed using IL-2 release from mature T cell clones as an indication of antigen specific activation (6). The role of IL-1β in thymocyte differentiation was then and continues to be implied by these results but not verified experimentally. Experimental results obtained in this study support previous reports which suggest that IL-1β is required for effective antigen presentation by TNCs during thymocyte development. We show TNCs to be capable of

interacting with immature $\alpha\beta TCR^{lo}CD69^-$ double positive thymocytes. A subset of the TNC-interactive population was rescued from apoptosis and the rescue activity was shown to be associated with the onset of Bcl-2 expression. Within the TNC-rescued population, a subset matured to the $\alpha\beta TCR^{hi}CD69^+$ phenotype only in the presence of IL-1β. These data suggest that TNCs may provide signals required for thymocyte rescue and maturation within the double positive stage of development. We propose that Bcl-2 expression is initiated through the thymocyte interaction with TNCs at the $\alpha\beta TCR^{lo}$ stage of development and that its expression may be necessary to maintain the viability of triple positive thymocytes with potential for positive selection.

MATERIALS AND METHODS

Isolation of Thymic Nurse Cells.

Thymi were removed from 2 week to 1 month old C57BL/6 mice. Thymic nurse cells were isolated using fetal calf serum density gradients as described by Werkele and Ketelson (1980). TNCs were allowed to release enclosed thymocytes and to form monolayers before exposure to tsA58 (originally obtained from Dr. P. Tegtmeyer) at a multiplicity of 10 PFU/cell at approximately 5×10^4 cells/ml in RPMI plus 2 μg/ml polybrene. After an adsorption period of 30 minutes, the cells were diluted with growth media and maintained in culture at 32°C before cloning by limiting dilution.

Transmission Electron Microscopy.

Freshly isolated thymocytes were co-incubated with a monolayer of tsTNC-1 cells overnight. The unbound thymocyte population was removed and the

remaining cells were fixed with 2% gluteraldehyde made in 1X phosphate buffered saline (PBS) for 1 hour at 4°C. The cells were then washed twice with 1X PBS, and treated with the secondary fixative, 1% osmium tetroxide, at room temperature for 30 minutes. The cells were rewashed in 1X PBS, removed from their surface and pelleted. Dehydration steps were done in ascending percentages of ethyl alcohol for five minutes, each terminating in three 10 minute treatments in 100% ethyl alcohol. A 1:1 dilution of 100% ethyl alcohol and propylene oxide was added to the pellet for 10 minutes, followed by a 30 minute incubation in 100% propylene oxide at room temperature. The cell pellets were then transferred to beam capsules and incubated in a 1:1 dilution of propylene oxide and epon 812 resin for 1 hour at room temperature, followed by an overnight incubation in 100% resin mixed with the polymerizer DMP-30. Each specimen block was then polymerized at 65°C overnight.

The specimen block was mounted in the LKB MT-2 microtome and sections were obtained using a blade angle of approximately 3°. Ultra-thin sections were collected using copper grids that were pre-washed in acetone. The sections were stained using a heavy metal double staining technique with 5% uranyl acetate and lead citrate. The grids were viewed using the Phillips 300 Transmission Electron Microscope at a voltage of 60V and photographs were taken using Kodak Technical Pan film.

Rescue Assay and FACS Analysis

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

preparation for FACS sorting. Thymocytes were resuspended at 5 X 10^6 cells/ml ^{3}H and CD4 CD8 double positive thymocytes were sorted (> 97 % pure) using a Colter Epics Elite (model F09102). 5 X 10^6 sorted thymocytes were incubated with 2 X 10^5 tsTNC-1 cells, or 3T3 cells for either 48 or 96 hours at the appropriate temperature. After incubation the thymocytes were removed and washed three times with RPMI, and analyzed for viability using forward versus side scatter. The cell surface phenotype for αβTCR, CD69, $H2^b$ and Ia^b antigens were also determined 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 exclusively to examine TNC-interactive thymocytes were performed using thymocytes that remained bound to TNCs after vigorous washing.

Proliferation Assay

The experimental group consisted of an equal concentration of fresh unsorted thymocytes cultured in flasks containing a monolayer of tsTNC-1 cells. The TNCs were grown overnight at a concentration of 1 X 10^5 cells/flask. Each time point consisted of three flasks. After 3 hours of co-incubation all unbound cells in suspension were removed with careful washes. At the end of each incubation period bound cells were removed with vigorous washes and counted. 1 X 10^6 cells were labeled per sample with 5 µl of ^{3}H-thymidine. Control samples consisted of thymocytes alone in culture. Three replicate flasks were prepared for each set of conditions. ^{3}H-thymidine was added to three flasks and washed immediately as a control for background counts. To each sample 5 ml of scintillation fluid was added and resuspended well. Counts per minute were obtained using a Wallac Rack Beta counter.

Proliferating Cell Nuclear Antigen (PCNA) Experiment

For each time period freshly isolated thymocytes (1 X 10^6 cells/ml) were added to monolayers of tsTNC-1 cells (1 X 10^5 cells/flask). All unbound thymocytes were removed with careful washes after 3 hours. The remaining cells were co-incubated for 12, 24 or 48 hours. At the end of each incubation period thymocytes were removed and stained with anti-Proliferating Cell Nuclear Antigen (PCNA). Briefly, cells were collected and washed once with 1X PBS. Cells were then fixed on ice using 1X PBS containing 2% paraformaldehyde for 1-2 hours. Cells were washed with 1X PBS and pelleted. Fixed cells were resuspended in 2 ml of permeabilization medium for 10-15 minutes on ice. Permeabilization medium consisted of 5 mM Hepes at a pH 7.5, 150 mM NaCl, 4% PBS and 0.1% Triton X-100. Cells were stained for 1-2 hours on ice in 200 µl of 1X PBS containing RNase A (0.5 mg/ml) and anti-PCNA-fluorescein conjugated at a concentration of 1:100. Cells were washed 3 times and analyzed using the FACS. The controls consisted of thymocytes incubated alone at a concentration of 1 X 10^6 cells/ml and thymocytes isolated the day of the FACS analysis.

Bcl-2 Staining

For each time period freshly isolated thymocytes (1 X 10^6 cells/ml in 5 ml) were added to monolayers of tsTNC-1 cells (1 X 10^5 cells/flask). All unbound thymocytes were removed with careful washes after 3 hours. The remaining cells were co-incubated for the appropriate time. Thymocytes obtained from these cocultures were washed three times with 1X PBS prior to staining, and spun at 3000 rpm in a table top centrifuge. The cells were then placed on ice in the residual volume of PBS. Cells were resuspended in 1 ml of ice cold

staining buffer [SB] (1% BSA, 0.1% saponin in 1X PBS, pH 7.4) and incubated for 15 minutes on ice. The samples were spun at 3000 rpm in a table top centrifuge before removing SB. Again the cells were resuspended in residual volume. Anti-Bcl-2 antibody (3F11 at 1 µl/ 10^6 cells) was added and incubated with cells for 45 minutes on ice, mixing cells every 10 minutes. The cells were washed 3 times in SB before adding the FITC conjugated secondary antibody (1:1000 dilution of anti-hamster FITC) and incubated for 45 minutes on ice. Finally, the samples were washed 3 times in SB followed by 1 wash with 1% BSA in 1X PBS and resuspended in 1 ml 1X PBS for FACS analysis.

TUNEL Assay

The TUNEL assay was performed according to the "*In Situ* Cell Death Kit, AP" by Boehringer Mannheim. TNC/thymocyte complexes were washed with 1X PBS and fixed with 4% paraformaldehyde and 0.1% Triton X-100 on ice for 2 minutes. 5 µl of TdT and 45 µl of fluorescein-labeled dUTP were added to the sample and incubated at 37°C for 60 minutes. The samples were washed with 1X PBS before analysis with a fluorescence microscope. The samples were then treated with 50 µl of anti-fluorescein antibody conjugated with alkaline phosphatase. After the substrate reaction, the cells were analyzed with a light microscope. Apoptotic thymocytes incorporated the stain in the nucleus. On slides, internal controls were the nuclei of the unstained thymocytes and that of the TNC. The samples were prepared on slides for visualization with a fluorescence microscope or in suspension for analysis using the FACS.

RESULTS

Internalization and Rescue of αβTCR$^+$Double Positive Thymocytes

We have previously demonstrated that our temperature sensitive SV40-transformed thymic nurse cell lines maintain the ability to both bind and internalize a subpopulation of the αβTCR^{lo}CD4$^+$CD8$^+$ thymocytes at 32°C but not at 38°C (16). A transmission electron micrograph of αβTCR$^+$ double positive thymocytes internalized *in vitro* at 32°C by a cell of the temperature sensitive line tsTNC-1 is shown in Fig. 1A. To determine the developmental function of the thymocyte/TNC interaction, 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 or 96 hours using forward versus side scatter to determine viability (Fig. 1B). Survivors were sparse in control samples after 96 hours of culture (3% viability of thymocytes with 3T3; 0.7% viability with tsTNC-1 at 38°C) but a relatively high frequency of viable cells was detectable in samples grown at 32°C in the presence of tsTNC-1 cells (43% viability). In a previous study, we showed that approximately 20 % of all double positive thymocytes bind TNCs. Assuming double positives make up 85 % of the total thymocyte population, 20 % bind and 40 % of those remain viable, then only about 6.8 % of the total population or 8 % of the double positive population remain viable after incubation with TNCs.

Selection during MHC restriction involves an interaction between the αβTCR and MHC antigens (12, 13). An earlier study revealed that antibodies against MHC class I and/or class II antigens do not interfere with the binding process between double positive thymocytes and TNCs (10). To determine

whether an interaction between the αβTCR and MHC antigens is required for the rescue activity described above, antibodies against both MHC antigens were used in long term TNC/thymocyte cocultures. Before coculture, the level of expression of class I and/or class II MHC by tsTNC-1 cells was analyzed (Fig. 2A). High level expression of both MHC class I and/or class II antigens was detected on the cell surface tsTNC-1 cells. These same antibody preparations were then used in long term co-incubation experiments to determine their effect on TNC rescue activity. As seen in Fig. 2B, the percent rescue was drastically reduced in tsTNC-1 cultures at 32°C containing antibodies against either class I or class II determinants. Rescue was reduced to a greater extent when antibodies to both class I and class II antigens were used simultaneously.

Since their discovery in 1981, TNC thymocytes have been described as proliferating cells when analyzed from *in vivo* preparations (1, 2). To determine the cell cycle status of the TNC-interactive thymocyte population in our *in vitro* system, we performed proliferation assays using ^{3}H-thymidine. Fig. 3A demonstrates that there is a subset of dividing cells among freshly isolated thymocytes and the population of cells that bind thymic nurse cells as evidenced by the comparable levels of ^{3}H-TdR CPM observed at 3 hours. With increasing time in culture the TNC-interactive population continues to proliferate while thymocytes alone did not. This observation is further supported by the relative increase in the number of PCNA expressing thymocytes in the TNC containing cultures over time (Fig. 3B). PCNA is activated and required during DNA replication (24). Taken alone the change in PCNA expression could represent an increase in the number of dividing cells. However, given that the ^{3}H-TdR uptake remains constant throughout the course of the experiment the more likely interpretation is that TNCs

prevent the death of the proliferating $CD4^+$ $CD8^+$ thymocytes while the nondividing cells die.

Apoptosis of TNC-Thymocytes

Both live and dead double positive thymocytes have been detected within TNC vacuoles (10, 17). In those earlier studies, apoptosis was defined using propidium iodide, DNA fragmentation ladders or microscopic phenotypic characteristics (chromatin condensation) as markers for death. None of these methods are definitive assays for determining apoptotic death. We used the newly developed TUNEL assay, which results in terminal deoxynucleotidyl transferase-mediated labeling of DNA with numerous strand breaks as an indication of apoptosis. When the internalized population was examined after an overnight incubation, both viable and apoptotic thymocytes were detected (Fig. 4A and B). The internalized population was analyzed at 24 hour intervals for 96 hours (Fig. 4C-F). With increasing time in culture, apoptotic thymocytes within the specialized TNC vacuoles began to lyse until the structure of intact cells was no longer discernible. DNA fragments released from lysed thymocytes were then detected in a linear arrangement within the cytoplasm of the TNC indicating total destruction of enclosed thymocytes and release from the specialized TNC vacuoles (Fig. 4F).

We then used the TUNEL assay to examine cocultured thymocytes released from their association with tsTNC-1 cells. Unlike the experiment reported in Fig. 4, which only examined the TNC-internalized population, this experiment was designed to analyze the effect of coculture on the entire TNC-interactive population. FACS-sorted double positives were exposed to cocultures as described above in Fig. 1. After a three hour binding period, unattached thymocytes were removed. The thymocytes remaining in

suspension after 3 days were reanalyzed using the TUNEL assay and FACS analysis. Fig. 5A shows apoptosis to be greatly reduced in the TNC-bound fraction at 32°C when compared to the population recovered from tsTNC-1 cultures grown at 38°C, the temperature at which binding does not occur, and in control 3T3 cocultures.

Bcl-2 Expression in TNC-Interactive Thymocytes

To determine the relationship between the thymocyte/TNC interaction and Bcl-2 expression, we analyzed thymocytes obtained from co-incubations after staining with 3F11 antibody (Fig. 6). Bcl-2 expression was detectable in a subset of the TNC-bound population within 24 hours (6.7%) of culture. At 72 hours, most of the viable thymocytes cocultured with tsTNC-1 cells at 32°C express the Bcl-2 protein (50.7%). Recent studies suggest that the expression of Bcl-2 permits increased longevity of developing thymocytes but cannot override negative selection (18). We analyzed thymocytes released from the TNC interaction for both Bcl-2 expression and apoptosis using the TUNEL assay (Fig. 5B). The results of this experiment show a subset of the Bcl-2 expressing population to be TUNEL positive as well (Fig. 5B, quadrant 2).

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, TNC-interactive double positive thymocytes were selected as a function of their binding capacity (19). Bound thymocytes were recovered after an initial binding period of 3 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 $\alpha\beta TCR^{lo}CD69^{-}$ double positive population (Fig. 7). 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 (Fig. 7A). An intermediate increase in CD69 expression levels was also detected (Fig. 7B). However, $\alpha\beta TCR^{hi}CD69^{hi}$ cells were not observed. As stated above, a previous study reported TNCs to be deficient for complete antigen presentation alone, but the deficiency could be corrected in the presence of the cytokine IL-1β (6). Using identical experimental conditions described above with the addition of IL-1β, a subset of $\alpha\beta TCR^{hi}$ (Fig. 7A) and $CD69^{hi}$ (Fig. 7B) thymocytes were clearly visible at 48 hours in TNC. Thymocytes with intermediate to high expression levels of both the αβTCR (Fig. 7C) and CD69 (Fig. 7D) were detectable within freshly isolated TNCs as well.

DISCUSSION

The results of the data reported here suggest that TNCs play a role in the process of thymic education. An increased viability or rescue from programmed cell death was detected in the TNC-interactive thymocyte population. The surviving population was shown to be proliferative (Fig. 3). Using the TUNEL assay, it was shown that the death of TNC thymocytes results specifically from the process of apoptosis (Fig. 4 and 5). Thymocytes that were not released from the specialized TNC vacuoles became apoptotic with time. These cells were destroyed and the cellular fragments appeared to be digested within TNCs by a yet to be described mechanism. These data are consistent with earlier reports which suggest that TNCs have the ability to maintain or increase the viability of a subset of its interactive thymocyte

population, while selecting another subset to die through the process of programmed cell death (16).

Although earlier studies showed TNCs to be capable of extending the life of a subset of its interactive population, this activity was not associated with cell surface changes indicative of positive selection (16). Positive selection is defined by an interaction between the TCR and the MHC that allows lymphocytes to continue differentiation (11, 12). Here, we showed the TNC rescue activity to involve an interaction between the TCR and the MHC (Fig. 2). In the absence of IL-1β, rescue was detectable, but surviving thymocytes maintained the immature $\alpha\beta TCR^{lo}CD4^{+}CD8^{+}CD69^{-}$ phenotype (16). It is clear from these data that the rescue activity of TNCs does not require IL-1β. During this IL-1β-independent interaction, rescued thymocytes began to express the Bcl-2 protein (Fig. 6). The number of Bcl-2 expressing cells increased within the rescued population for up 72 hours of culture. These results draw a strong correlation between Bcl-2 expression and the rescue activity of tsTNC-1 cells.

In an earlier report, IL-1β-independent survivors were shown to express high levels of both CD4 and CD8 (16). $CD4^{hi}CD8^{hi}$ expressing cells have been shown by others to be associated with increased viability (11, 20). A $CD4^{hi}CD8^{hi}Bcl\text{-}2^{+}$ thymocyte subset was shown to exist in the normal mouse thymus, but their developmental potential remains unclear (21). Studies of this transitional phase during the double positive window of development, from low through the intermediate stage to high level expression of the αβTCR, have been difficult to perform *in vitro* because it is accompanied by massive cell death, antibodies to the αβTCR alone induce the shift to the $\alpha\beta TCR^{hi}$ phenotype, and only the $\alpha\beta TCR^{hi}$ population have developmental potential in culture (22). Analyses of this transitional phase have been improved recently using thymocytes isolated from *bcl-2* transgenic mice (23).

While thymocytes isolated from normal animals die quickly in culture (within 48 hours), sorted double positive subsets isolated from *bcl-2* transgenic mice remained viable from 4 to 6 days in culture (a lifespan that is equivalent to that found in association with tsTNC-1 cells in culture, ref. 16). A comparative study of the *in vitro* differentiation potential of $\alpha\beta TCR^{lo}$ and $\alpha\beta TCR^{hi}$ double positive subsets isolated from *bcl-2* transgenic mice showed only the $\alpha\beta TCR^{hi}$ population to have the capacity to give rise to mature single positive thymocytes. The *bcl-2* transgenic $\alpha\beta TCR^{lo}$ double positive population was shown to be capable of maturation only after intrathymic injection into semi-congenic recipients, suggesting a need for unknown thymic requirements not present in the *in vitro* system. It is possible that TNCs supply the requirements needed for a selected subset of double positive thymocytes to survive from the $\alpha\beta TCR^{lo}$ to the $\alpha\beta TCR^{hi}$ stage of development while selectively inducing apoptosis in the remaining fraction.

Our data suggest that the rescue signal provided by TNCs includes the activation of Bcl-2 expression (Fig. 6). We showed a subset of the TNC-rescued population to increase the level of expression of both the αβTCR and CD69 to become $\alpha\beta TCR^{hi}CD69^{hi}$ expressing cells (Fig. 7). Although intermediate increases of both αβTCR and CD69 were observed without the addition of IL-1β, maturation to the $\alpha\beta TCR^{hi}CD69^{hi}$ phenotype was shown to be dependent on the presence of IL-1β (Fig. 7A and B). Intermediate to high expression levels of both αβTCR and CD69 were also detected on thymocytes obtained from freshly isolated TNCs (Fig. 7C and D). We propose that Bcl-2 expression may be initiated through the thymocyte interaction with TNCs at the $\alpha\beta TCR^{lo}$ stage of development, and that its expression may be necessary to maintain the viability of thymocytes with potential for positive selection during MHC restriction (Fig. 8). On the other hand, TNC activation of Bcl-2 expression and

expression of the $TCR^{hi}CD69^{hi}$ phenotype does not prevent (Fig. 5B) negative selection nor do they ensure maturation into the single positive stage of development.

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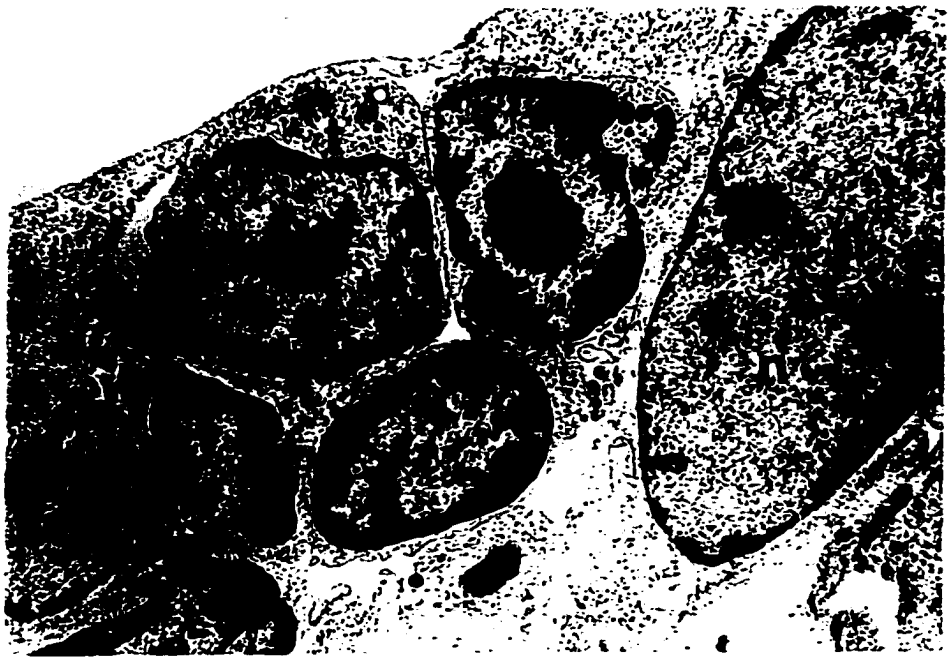
FIGURES AND LEGENDS

Fig. 1. (A) Transmission electron micrograph of a thymocyte/tsTNC-1 cell complex at 32°C, (t) labels one of the internalized thymocytes and (n) is placed over the nucleus in a tsTNC-1 cell. (B) TNC Rescue Assay. Freshly isolated thymocytes were stained with antibodies against CD4 and CD8 and sorted to a purity of over 97%. Sorted double positive thymocytes (5×10^6) were incubated with 2×10^5 tsTNC-1 at 32°C or 38°C, or with 2×10^5 3T3 cells at 32°C. Cocultures were maintained for either 48 hours or 96 hours at the appropriate temperature. All remaining thymocytes either bound or in suspension were recovered and reanalyzed for viability using FACS forward versus side scatter. The percentage reported is the average of three independent experiments. Less than 1% of the thymocyte population were viable after 96 hours of culture in which 3.0 μm cyclopore membranes were used to prevent contact of thymocytes with TNCs at 32°C (data not shown). 3T3 cultures were incubated at 32°C.

A



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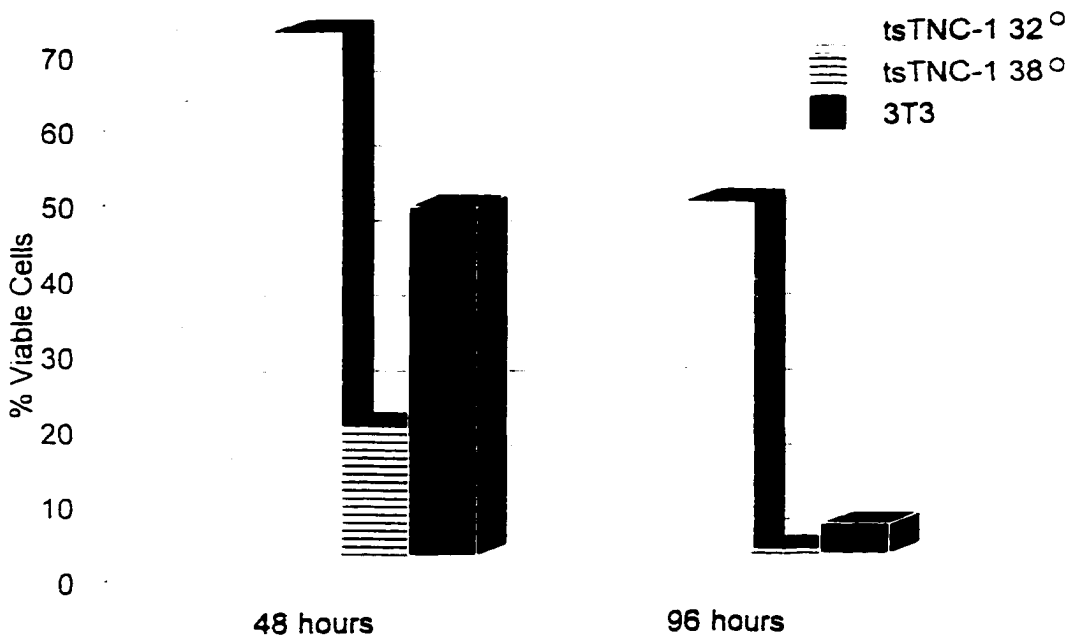
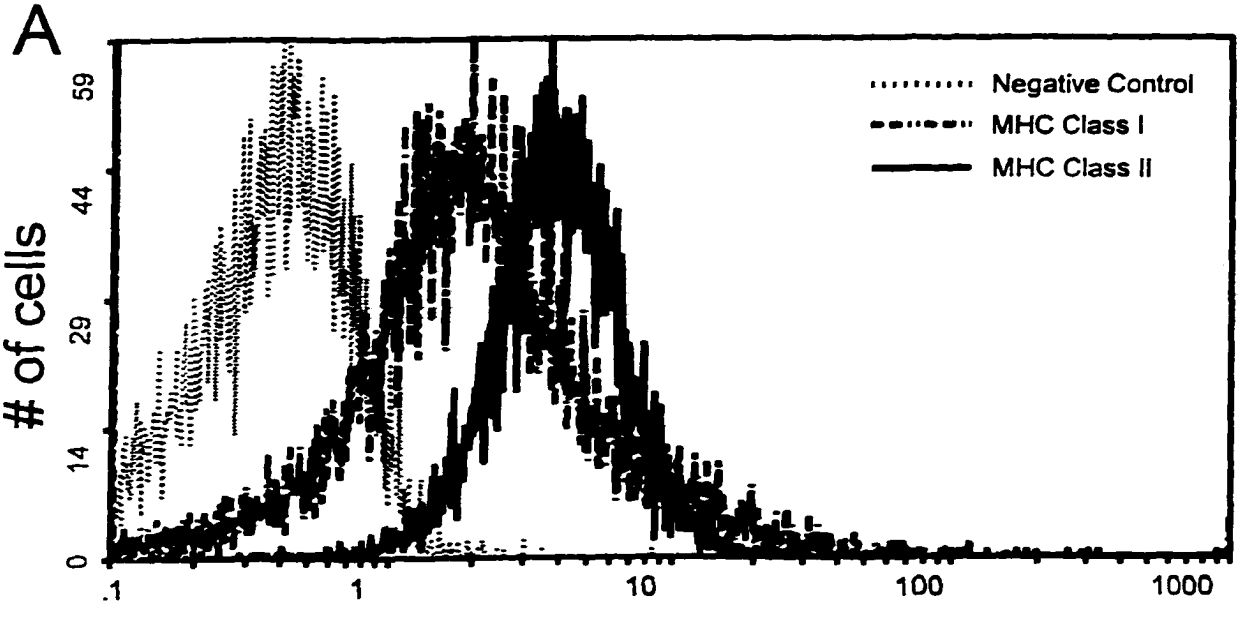


Fig. 2. (A) TsTNC-1 cells were stained with antibody preparations against MHC class I ($H2K^b$) and class II (Ia^b) before FACS Colter (model Elite) analysis. (B) Thymocytes were resuspended at 5 X 10^6 cells/ml and double positive thymocytes were sorted using a FACS as described above. Sorted thymocytes (5 X 10^6) were co-incubated with 2 X 10^5 tsTNC-1. Cocultures were maintained for 48 hours at 32°C without antibody or with the antibody preparation shown beneath each column. All remaining thymocytes either bound or in suspension were recovered and reanalyzed for viability using FACS forward versus side scatter. The numbers represent the percentage of cells within the viable gate. Another control included antibody to either class I or class II MHC antigens in thymocyte only cultures. No effect on thymocyte survival was detected.



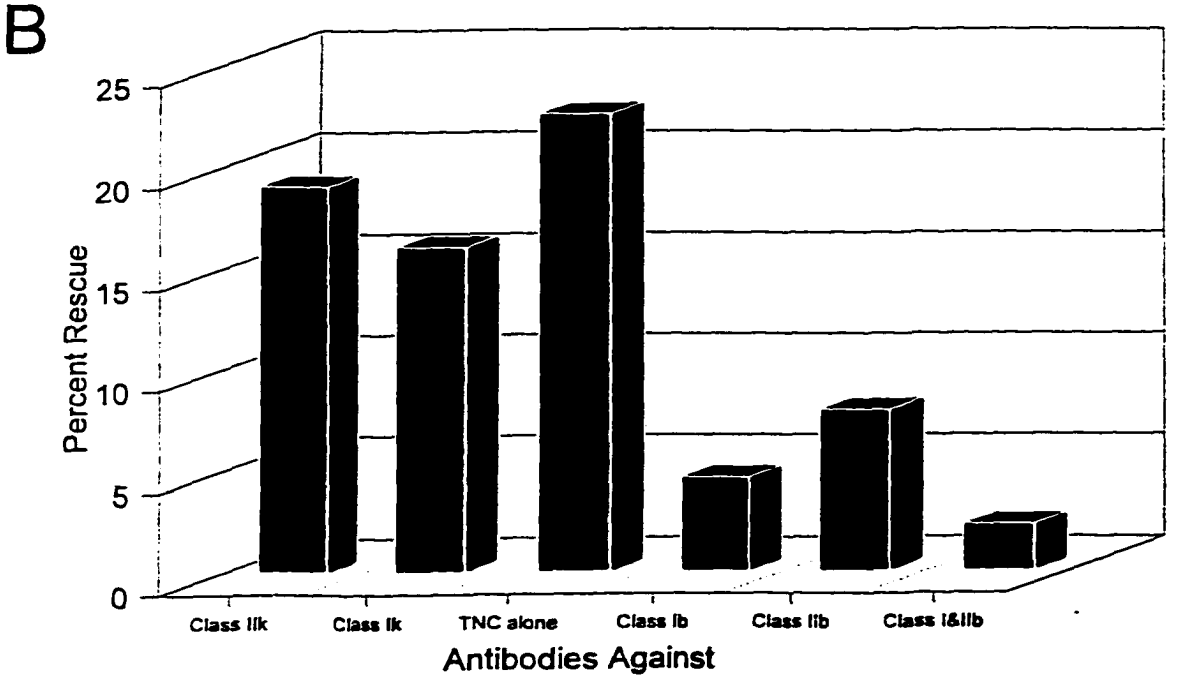
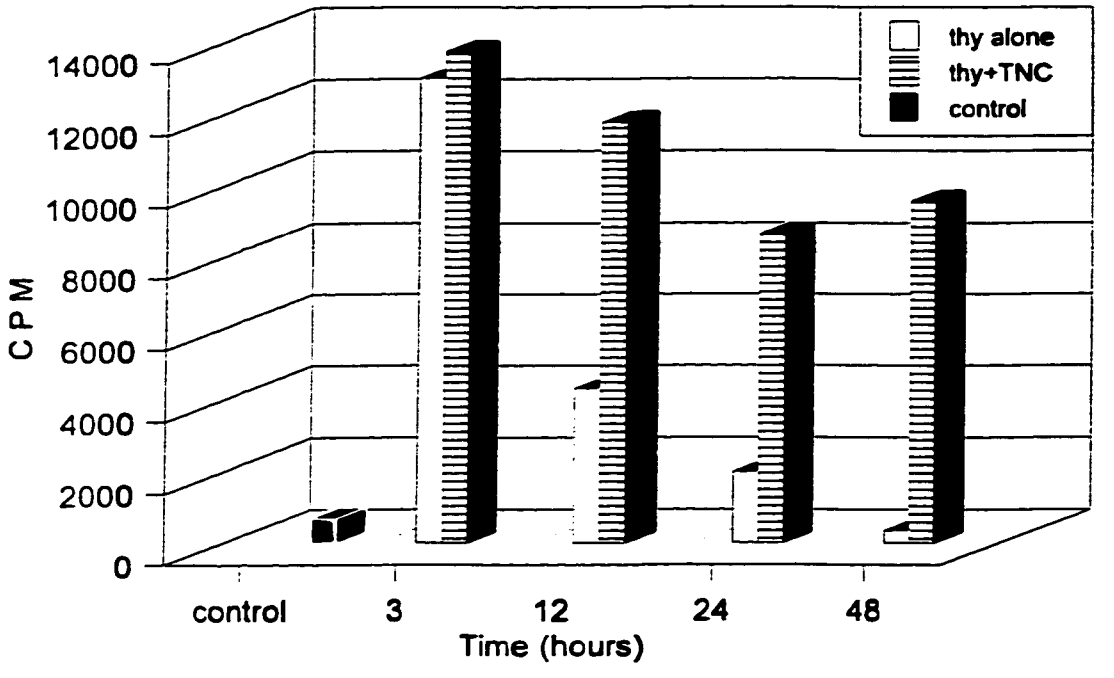


Fig. 3. (A) Thymocytes were recovered from tsTNC-1 cocultures and labeled with ^{3}H-thymidine at the appropriate times. The percent uptake of tritium was determined in cultures containing thymocytes alone (thy alone), or cocultures of thymocytes and tsTNC-1 cells (thy + TNC) at 32°C. The control group consisted of fresh unsorted thymocytes alone to which ^{3}H-thymidine was added and washed away immediately. (B) For each time period freshly isolated thymocytes were added to flasks containing 1 X 10^{5} TNCs and pre-incubated for 3 hours. All unbound thymocytes were then removed with careful washes. A sample of thymocytes which had been incubated without tsTNC-1 cells as well as the bound population from one flask incubated with tsTNC-1 were analyzed at three hours to determine the initial expression of Proliferating Cell Nuclear Antigen (PCNA). The remaining cultures were co-incubated for 12, 24 or 48 hours. At the end of each incubation period bound cells were removed and stained with FITC conjugated anti-PCNA. The thymocytes were then analyzed using FACS. The controls consisted of thymocytes incubated alone (thy alone) over the time listed and fresh thymocytes isolated the day of FACS analysis (fresh thy).

A



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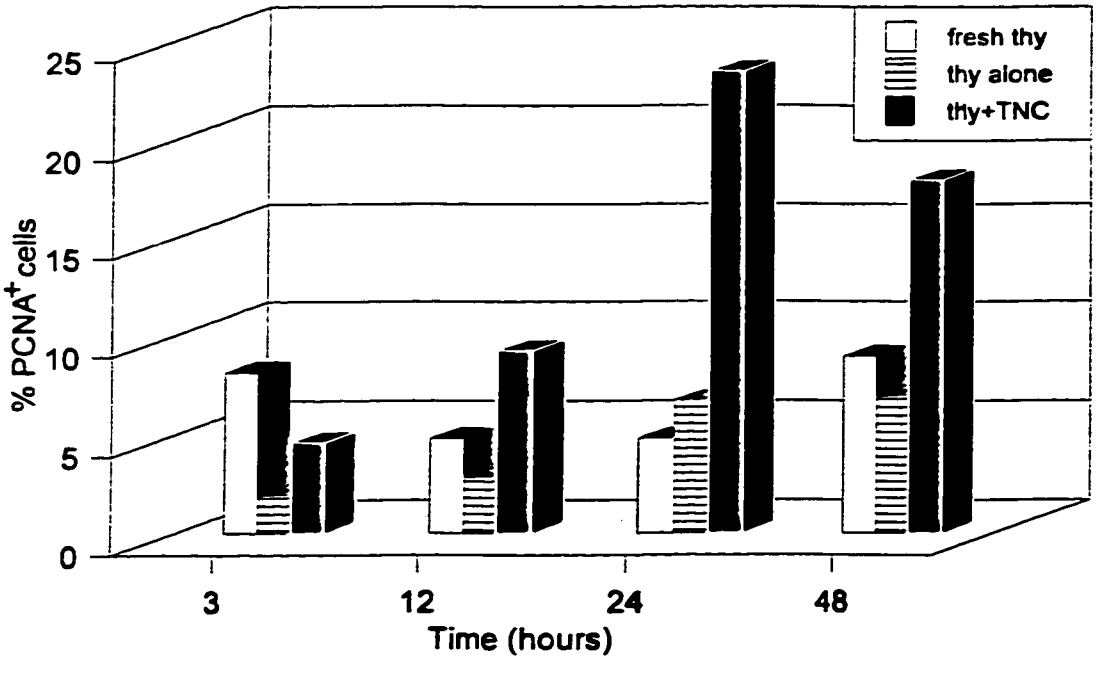


Fig. 4. The fluorescence TUNEL assay was performed using samples isolated from cocultures of thymocytes and tsTNC-1 cells after an overnight incubation period. The thymocytes that remained internalized after thorough washings were visualized using a fluorescence microscope, (A) phase and (B) fluorescence micrographs. Cocultures were then allowed to incubate for (C) 24, (D) 48, (E) 72 or (F) 96 hours before analysis using the HRP-TUNEL assay.

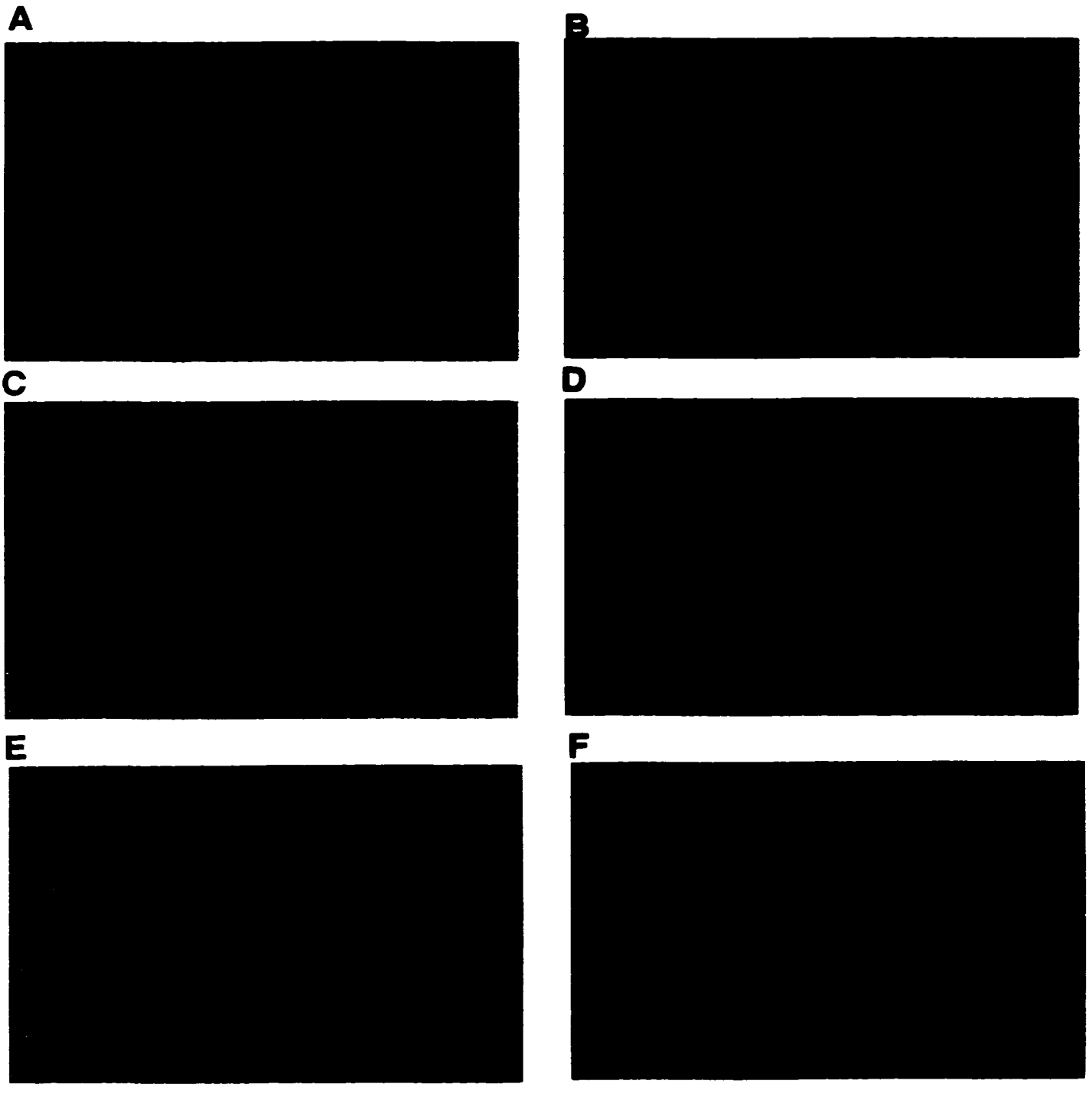
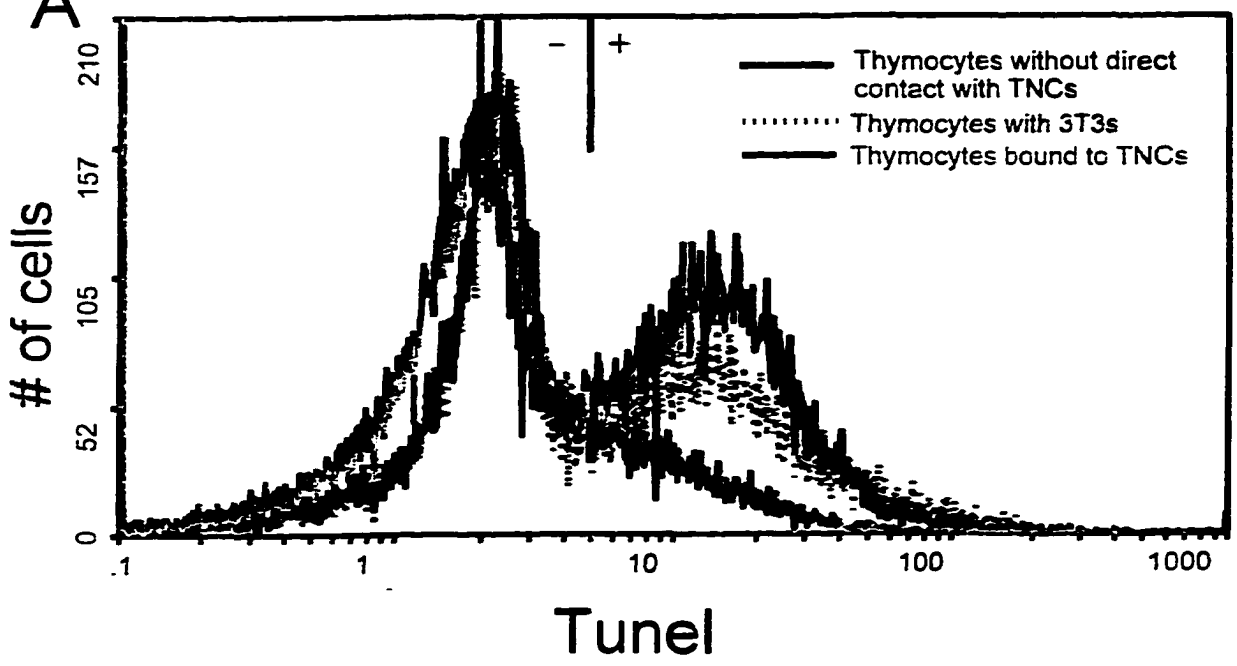


Fig. 5. (A) FACS-sorted double positives were exposed to cultures of tsTNC-1 cells as described in the Materials and Method section. After a three hour binding period, unattached thymocytes were removed. Thymocytes obtained after three days of coculture with tsTNC-1 cells were stained using the fluorescence TUNEL method and analyzed with the FACS. Thymocytes were cultured with (Thymocytes bound to TNCs) or without (Thymocytes with 3T3s at 32°C) a monolayer of tsTNC-1 cells, or incubated with tsTNC-1 cells at 38°C (Thymocytes without direct contact with TNCs). Stained cells fall to the right of the + gate. (B) Thymocytes collected after 72 hour coculture with tsTNC-1 cells were stained with 3F11 antibody and prepared using the fluorescence TUNEL assay before FACS analysis.

A



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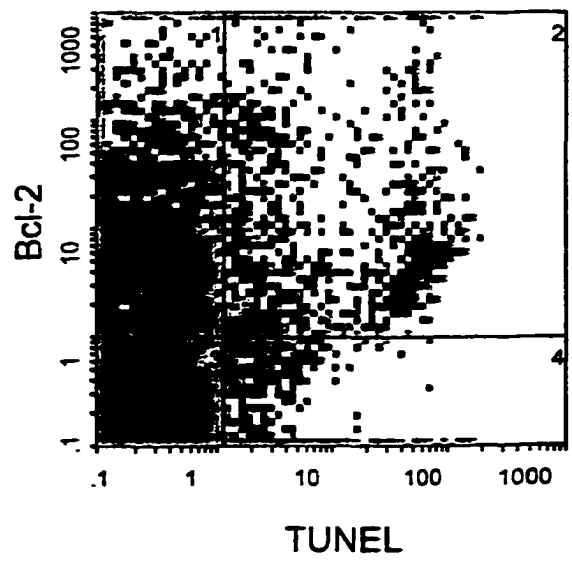


Fig. 6. Thymocytes alone in culture (thy alone), thymocytes cocultured with 3T3 cells at 32°C (3T3), unbound thymocytes removed after a three hour binding period and placed in culture alone (unbound thy) and thymocytes that remained in association with tsTNC-1 cells after these initial washes (TNC bound) were stained with the 3F11 antibody before analysis with the FACS. All recoverable thymocytes were analyzed after 6, 24, 48 or 72 hours of incubation. $FITC^-$ samples were incubated with fluoresceinated second antibody only.

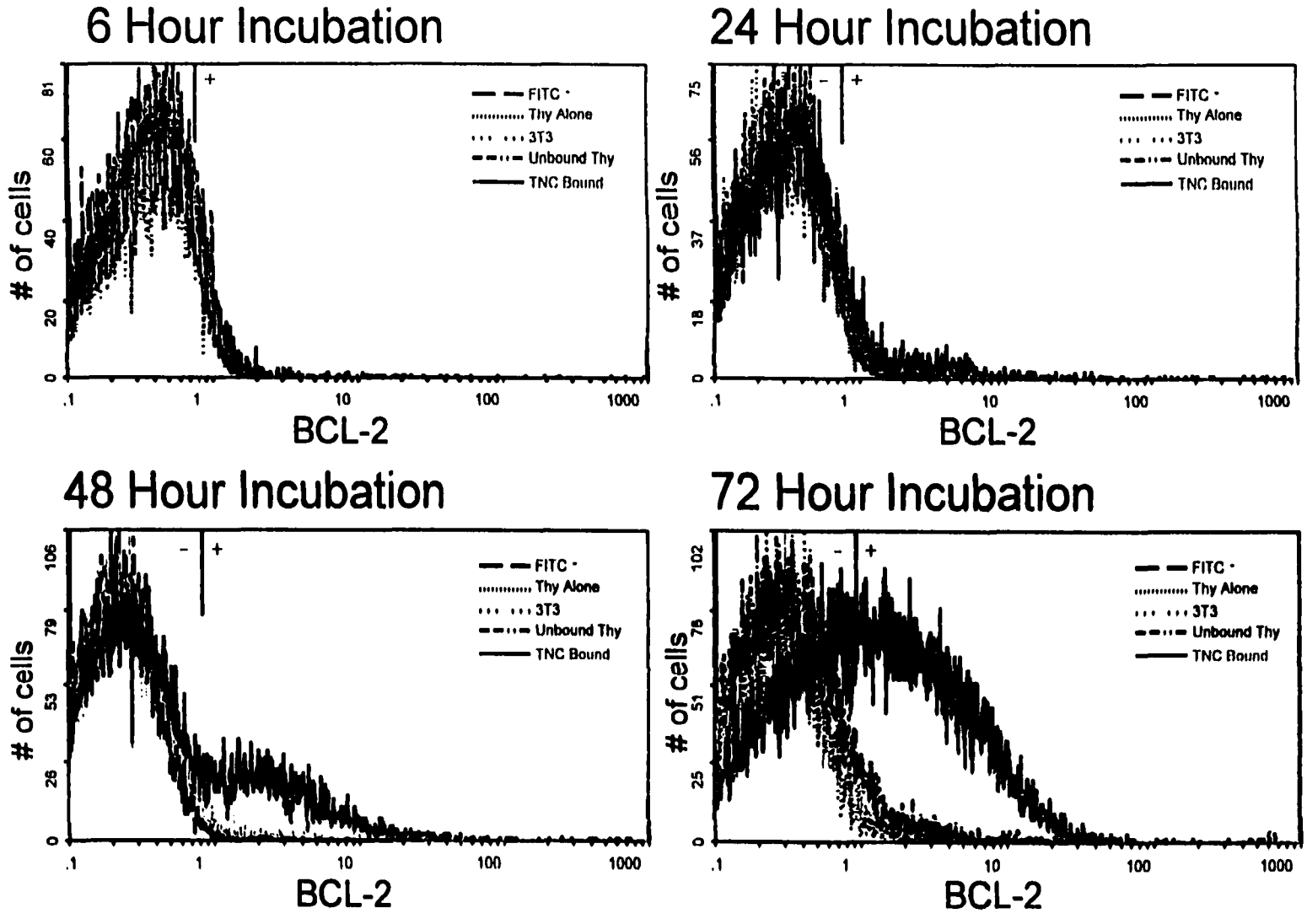


Fig. 7. Freshly isolated thymocytes were exposed to monolayers of tsTNC-1 cells for 3 hours. Nonadherent thymocytes were removed by washing with PBS and the bound population was recovered and analyzed for the cell surface expression levels of the αβTCR and CD69 after a 48 hour incubation period. (A) FACS analysis of αβTCR levels on thymocytes obtained from tsTNC-1 cocultures with or without the addition of IL-1β. One control consisted of thymocytes bound to tsTNC-1 cells and analyzed after the initial three hour binding period (thymocytes initially bound). $FITC^-$ samples were incubated with fluoresceinated second antibody only. (B) FACS analysis of CD69 levels on thymocytes obtained from tsTNC-1 cocultures with or without the addition of IL-1β. One control consisted of thymocytes bound to tsTNC-1 cells and analyzed after the initial three hour binding period (thymocytes initially bound). $FITC^-$ samples were incubated with fluoresceinated second antibody only. (C) FACS analysis of αβTCR levels on the cell surfaces of thymocytes released from freshly isolated TNC. The control sample is representative of the entire thymocyte population. (D) FACS analysis of CD69 levels on the cell surfaces of thymocytes released from freshly isolated TNC. The control sample is representative of the entire thymocyte population.

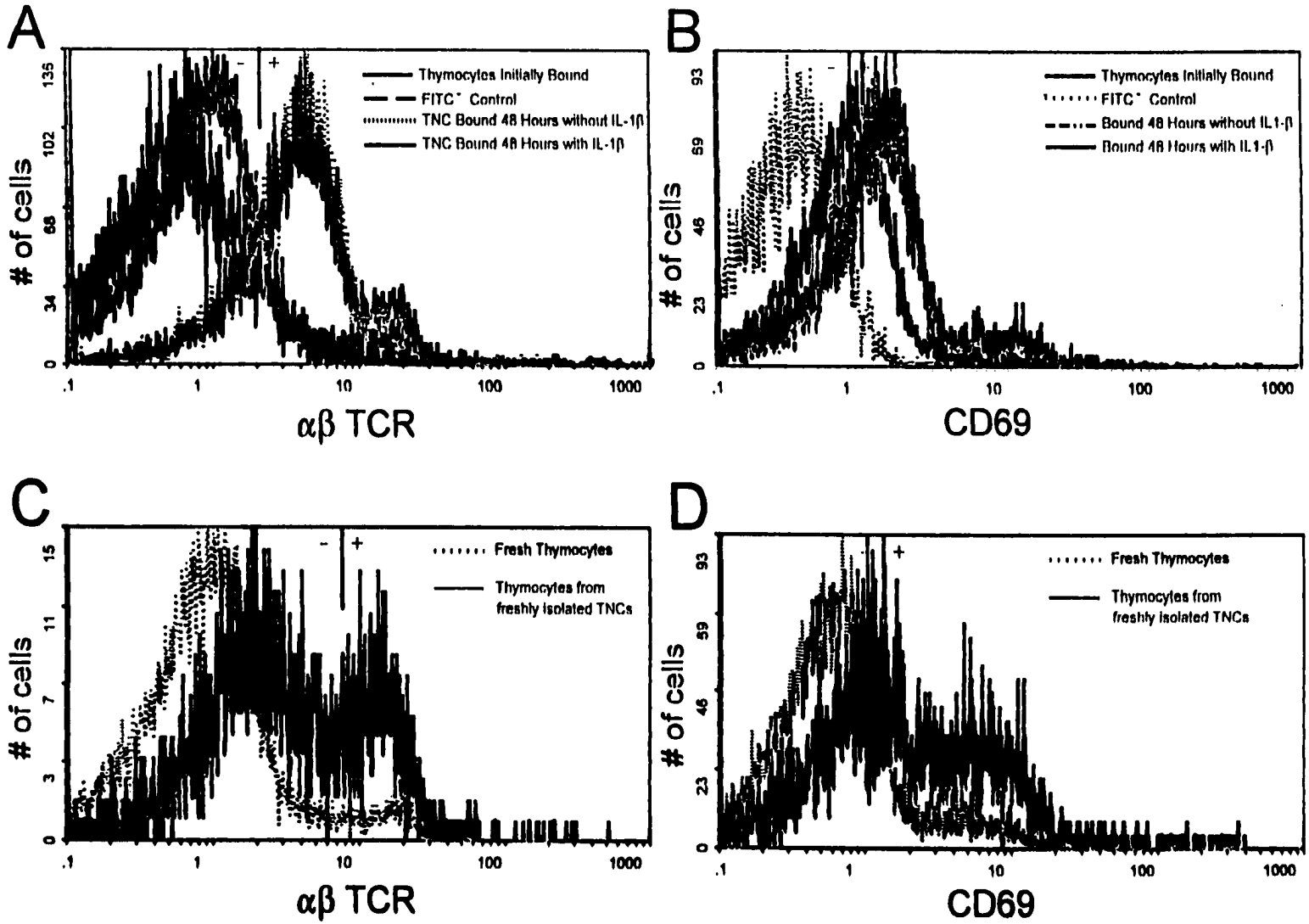
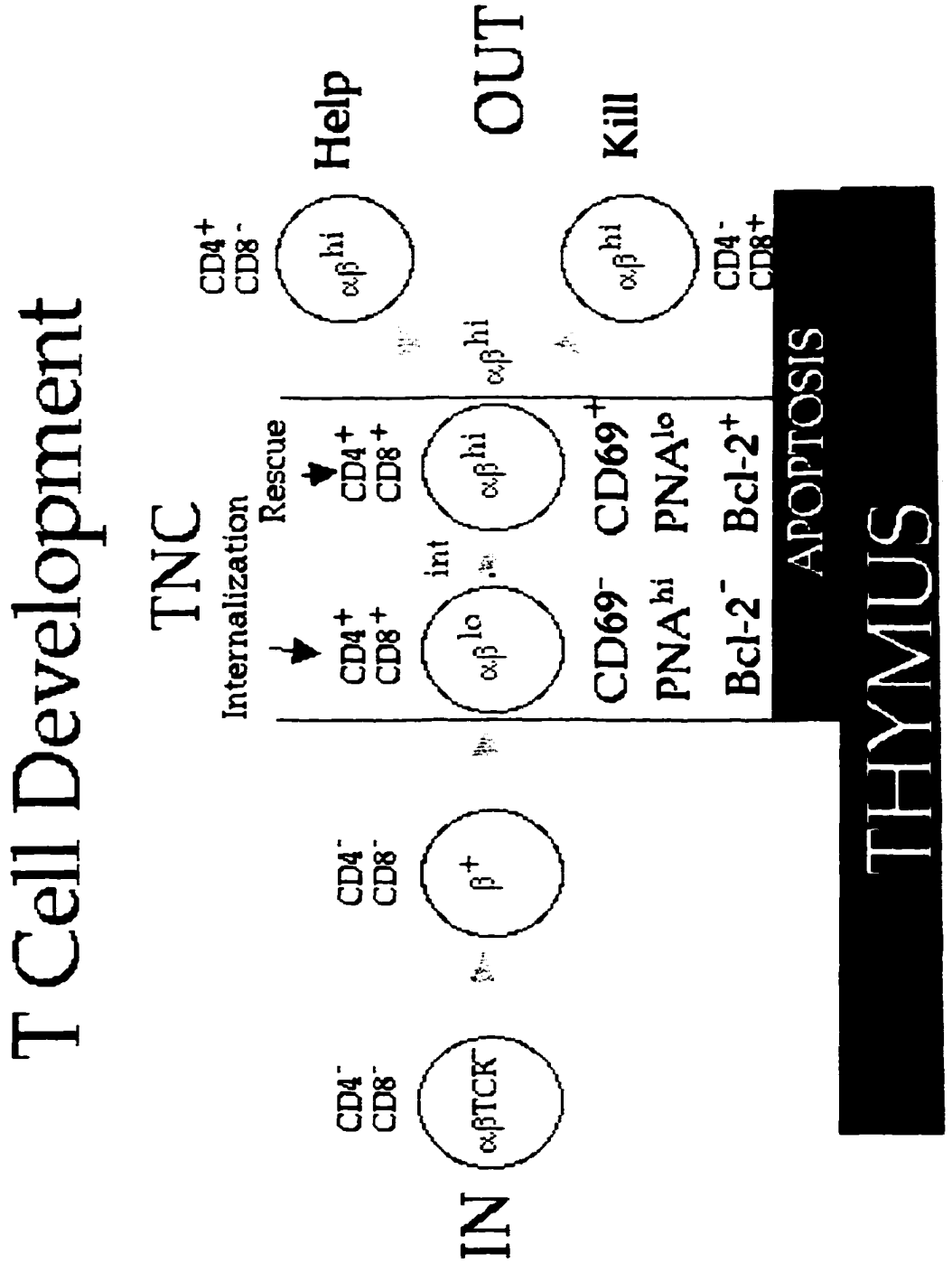


Fig. 8. A diagram of the T cell developmental pathway which includes processes that result from the thymocyte/TNC interaction.



CONCLUSIONS

The goal of this study has been to determine the functional role of TNCs in T cell development. TNCs are one of two known mammalian cell types that produce structures that completely enclose developing cells within cytoplasmic compartments during their development; the other being Sertoli cells involved in spermatogonia uptake in sperm cell development (Russell, LD, 1980; Russell, LD and Peterson, RN, 1985). The development of immortalized TNC lines provided an ideal model system to study how the internalization event occurs, the selective nature of the thymocyte-TNC interaction and cell fate as a function of this interaction.

As described in chapters 1 and 2, thymocyte binding and internalization by TNCs occurs via membrane ruffling and is a selective process. Using long-term video and scanning electron microscopy, the binding and internalization process was shown to occur within a 6 hr period and results in thymocytes that are internalized in vacuoles within the cytoplasm of the TNC. Until recently, attempts at blocking this event were unsuccessful **(Li, et. al., 1992)**. Thymocyte interaction with TNCs appears to involve a membrane-bound peptide that is TNC-specific. Recent studies in our laboratory have shown that such a protein (47 kd in size) is involved in the identification of TNC interactive thymocytes (Pezzano, et. al., 1998). The development of the first TNC specific antibody allowed us to identify and isolate this protein for future study.

The thymocyte\TNC interaction has been shown to involve a subpopulation of immature thymocytes that display a $CD4^+CD8^+\alpha\beta TCR^+$ phenotype; a phenotype identical to that of cells involved in MHC restriction. The creation and development of temperature-sensitive (ts) TNC lines

provided the necessary controls for studying cell fate as a function of their interaction with TNCs. Examination of thymocyte viability and the expression of early activation markers revealed that, at the permissive temperature for viral replication, only a fraction of these thymocytes were rescued from apoptosis. Thymocytes in that rescued population also showed shifts in expression of early maturation markers indicative of cell maturation. Antibody blocking studies performed with MHC class I and class II on the TNC surface did not prevent thymocyte\TNC interactions from occurring. It did, however, impede thymocyte rescue thereby demonstrating the necessity of the MHC-TCR interaction for rescue. The surviving cell population displayed a phenotype identical to that of thymocytes following MHC restriction. With this evidence in hand, it can be said that TNCs play an important role in MHC restriction.

Further support of this theory is shown in chapter 3. Developing thymocytes show changes in the expression of the cell surface markers CD69 and PNAr and receptor αβTCR when the lymphokine IL-1β is present during their interaction with TNCs. Full antigen expression on the surface of TNCs is required for thymocytes to display a more mature phenotype as a result of MHC restriction. Although the presence or absence of IL-1β in cocultures had no effect on thymocyte rescue, it was required for an increased level of mature expression of thymocytes in the rescued cell population. The results seen in the presence of this lymphokine mirror those seen *in vivo*.

Regardless of the presence or absence of IL-1β in cocultures, Bcl-2 expression was initiated through interaction of TNCs with DP thymocytes that have low level expression of both αβTCR and CD69. In spite of its know capabilities for prolonging the life of select thymocytes, the expression of Bcl-2 does not prevent negative selection from occurring (Gratiot-Deans, et. al.,

1993; Strasser, et. al., 1994; Veis, et. al., 1993). It also does not guarantee the maturation of thymocytes into SP cells. Upon closer examination of the known expression pathway followed by Bcl-2 during T cell development in the thymus, our findings present yet another avenue of thought on what is known to occur at the DP stage in development. It is known that Bcl-2 expression is down regulated at the DP stage of T cell development. It is up regulated during positive selection and persists of mature T cells in the periphery **(Linette, et. al., 1994; Tao, et. al., 1994)**. However, the data reported in this study show thymocytes that interact with TNCs display a DP phenotype, and initiate Bcl-2 expression at this stage in development.

Not only are TNCs responsible for thymocyte rescue and apoptosis, they carry out the unique task disposing of apoptotic cells (Pezzano, et. al., 1996). Using a modified TUNEL assay, the viability of internalized thymocytes was monitored over a 96 hr period. Cells marked to undergo apoptosis remain within the vacuoles of the TNCs and degrade without ever being released. This process clearly mimics negative selection. Comparable results were shown *in vivo* (Appendix, Fig. A-1, Fig. A-2). Lysosomes were found in close proximity to these vacuoles (Samms, et. al., 1999). Using confocal microscopy, we show fusion of vacuoles containing apoptotic thymocytes with lysosomes. With time, these apoptotic cells were shown to be degraded.

The research performed in this thesis show thymic nurse cells play an important role in T cell development. The phenotype displayed by thymocytes that interact with TNCs and the events that occur as a function of their interaction with TNCs suggest that these cells contribute to MHC restriction. TNCs are believed to be involved in both positive and negative selection. The development of our TNC lines supplies an ideal model system

for further studies of T cell development. Future study objectives related to this project include the investigation of signal transduction factors involved in the activation of thymocyte maturation and selection as a result of the thymocyte\TNC interaction. A thorough investigation of the apoptotic events occurring within the vacuoles of the TNCs would also prove informative. Another useful study would be to investigate the effects the addition of TNCs to a TNC-deficient or autoimmune mouse. Examining the contributions of TNCs in animals that display autoimmunity a crucial study to determine the true role of TNC in T cell development.

APPENDICES

Fig. A-1. Thymic nurse cells were isolated after mechanical dispersion of the mouse thymus. The resulting TNCs were analyzed using the TUNEL assay to detect apoptotic thymocytes within the TNC cytoplasm. Freshly isolated TNCs are shown using phase microscopy in the left panel and fluorescence microscopy in the right panel. The same cell shown in phase in the left panel is shown under fluorescence in the right panel. Apoptotic thymocytes within TNCs show fluorescence in the right panel. Two TNCs are shown to demonstrate variability of thymocyte apoptosis between TNCs. Fluorescein-labeled dUTP alone was added to the cells as a control and is shown in Figures E and F.

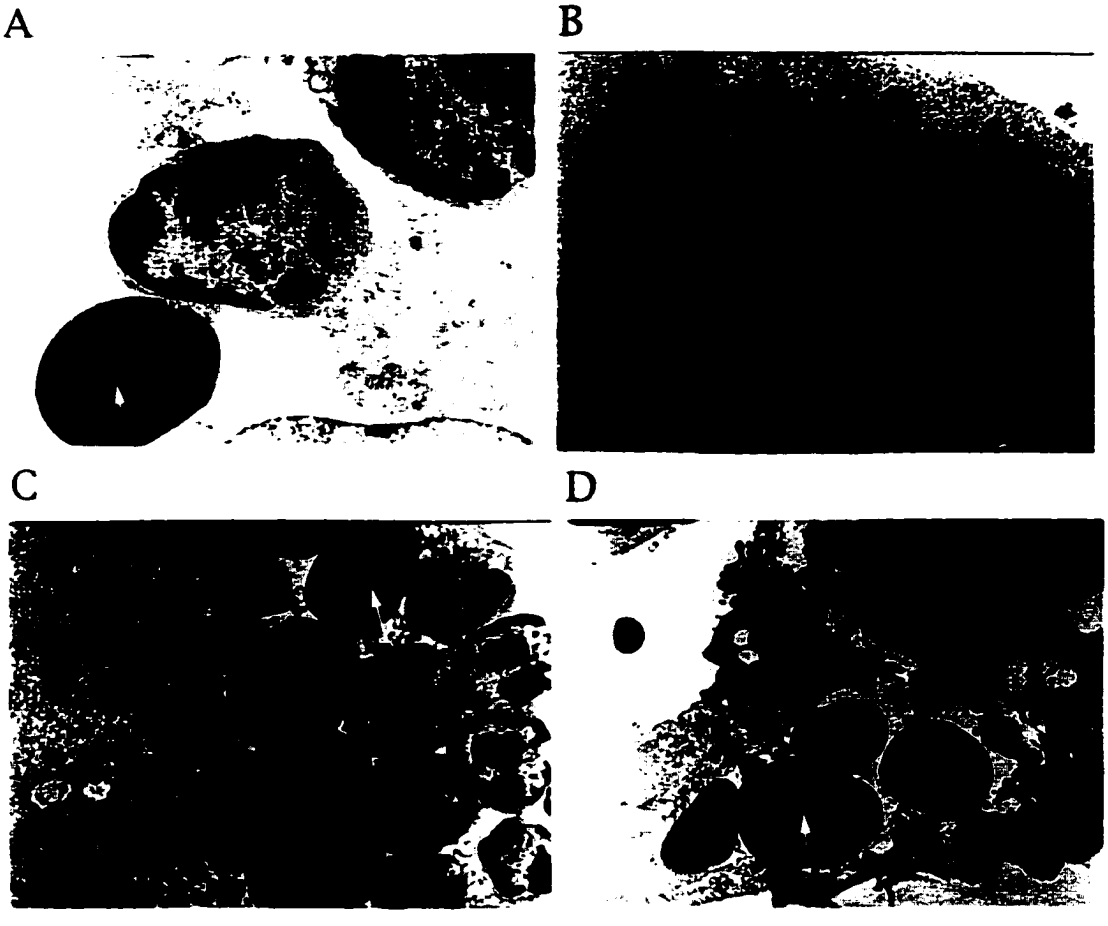
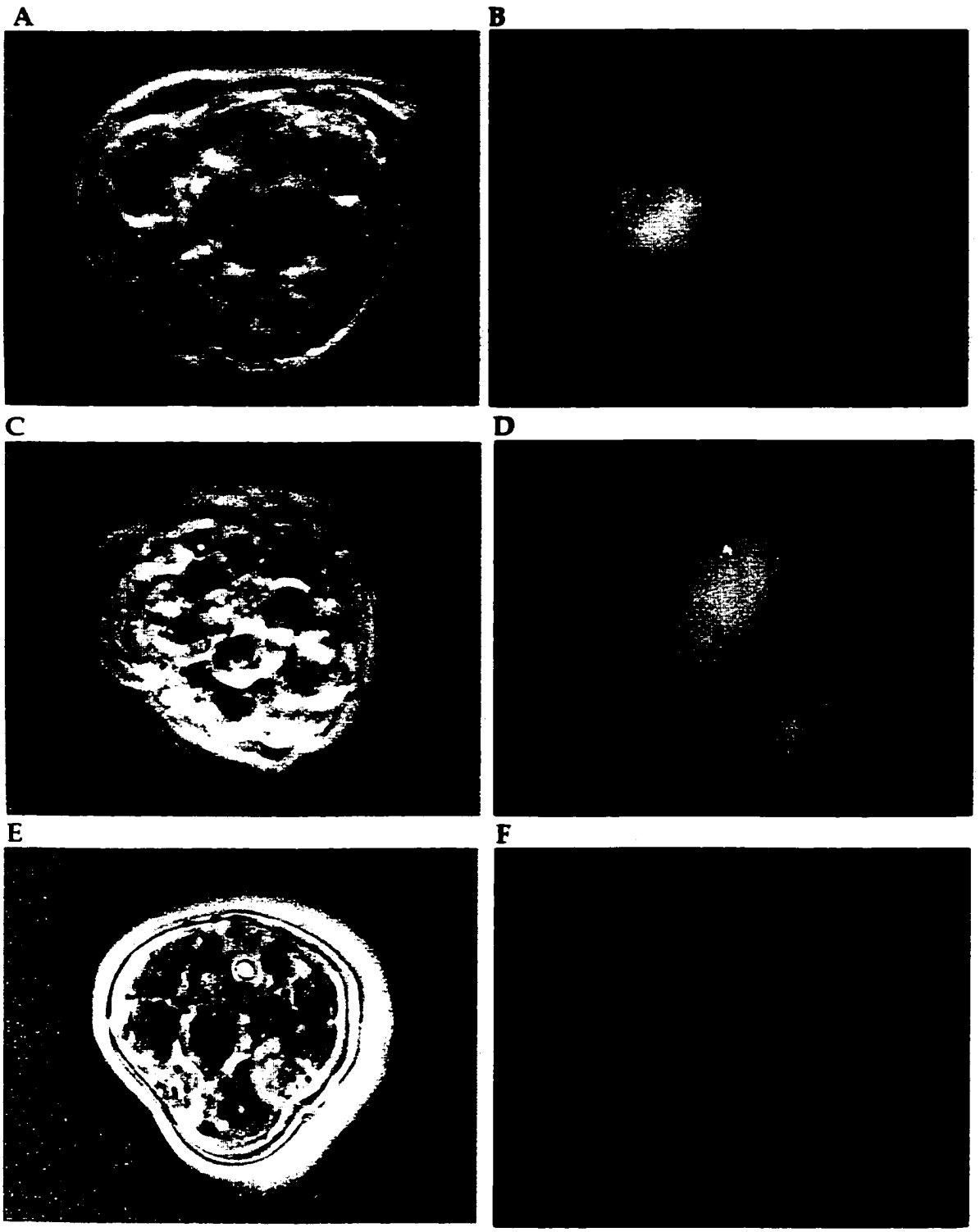


Fig. A-2. TEM of internalized thymocytes. (A) TNC-internalized thymocytes. The arrow indicates an apoptotic thymocyte. Magnification $6.8X10^6$. (B) Thymic nurse cell before exposure to thymocytes. Magnification 10^4. (C) Cytoplasmic thymocytes 2 hours after internalization. Magnification 10^4. (D) Cytoplasmic thymocytes 10 hours after internalization. Magnification 10^4.



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