The GTPase Rho Controls a p53-dependent Survival Checkpoint during Thymopoiesis

By Patrick S. Costello, Steve C. Cleverley, Ricciarda Galandrini, Stefan W. Henning, and Doreen A. Cantrell

From the Lymphocyte Activation Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom

Abstract

During the early stages of thymopoiesis, cell survival is controlled by cytokines that regulate the expression of antiapoptotic proteins such as Bcl-2. At the pre-T cell stage, a critical checkpoint for β chain selection is monitored by the tumor suppressor p53: pre-T cells can survive and differentiate when p53 is removed genetically or when its proapoptotic function is inactivated physiologically as a consequence of signaling through the pre-T cell receptor complex. Previous work has shown that the guanine nucleotide binding protein Rho controls cell survival in T cell progenitors. Here we define the survival pathways controlled by Rho in pre-T cells and show that this GTPase is a pivotal regulator of the p53-mediated checkpoint operating at the time of β selection: loss of Rho function results in apoptosis in pre-T cells, but this cell death is prevented by loss of p53. The prevention of cell death by loss of p53 restored numbers of early T cell progenitors but did not fully restore thymic cellularity. Further analysis revealed that loss of Rho function caused survival defects in CD4/8 double-positive thymocytes that is independent of p53 but can be prevented by ectopic expression of Bcl-2. These studies highlight that the GTPase Rho is a crucial component of survival signaling pathways in at least two different thymocyte subpopulations: Rho controls the p53 survival checkpoint in pre-T cells and is also crucial for a p53 independent survival signaling pathway in CD4/8 double positives.

Key words: pre-T cell • signaling • development • apoptosis • thymus

Introduction

The development and maturation of T lymphocytes in the thymus is an essential process for the formation of the peripheral immune system. The progression of T cells through the early stages of intrathymic differentiation may be defined by the sequential pattern of expression of a number of surface markers, including CD44 and CD25 (for review see references 1–3). Initially, cells are $CD44^+$ $CD25^-$ (termed DN1); they then go on to acquire CD25 (DN2), when they become committed to the T cell lineage and begin to rearrange TCR β loci. The cells then lose CD44 expression and continue β chain rearrangements to completion (DN3). At the DN3 pre-T cell stage, cells that successfully rearrange their TCR β locus and express a functional receptor complex known as the pre-TCR complex proliferate rapidly, downregulate CD25, and differentiate into DN4 cells (3–5). At the next developmental stage, cells coexpress the receptors for MHCs, CD4 and CD8, and within the CD4/8 double-positive (DP)[1] compartment undergo TCR α chain rearrangements. Upon expression of a functional pre-TCR complex, cells are subjected to the processes of positive and negative selection (for review see references 6 and 7) that ensure the generation of mature CD4 or CD8 single-positive (SP) T lymphocytes that exit to the peripheral lymphatic organs. In the thymus, the ordered acquisition of antigen receptors and cytokine receptors allows T cells to survive and progress to the next developmental stage. Any genetic manipulation that interferes with thymocyte survival mecha-

P.S. Costello and S.C. Cleverley contributed equally to this work.

R. Galandrini's present address is Dept. of Experimental Medicine and Pathology, University of Rome La Sapienza, V.le R. Elena, 324 Rome, Italy.

S.W. Henning's present address is Dept. of Molecular and Cell Biology, University of California, 415 Life Science Addition, Berkeley, CA 94720.

Address correspondence to Doreen A. Cantrell, Lymphocyte Activation Laboratory, Rm. 105, Imperial Cancer Research Fund, 44 Lincolns Inn Fields, London WC2A 3PX, UK. Phone: 44-020-7269-3231; Fax: 44-020-7269-2831; E-mail: d.cantrell@icrf.icnet.uk

[1]*Abbreviations used in this paper:* DP, double-positive; RAG, recombinase-activating gene; SP, single-positive.

nisms has a severe impact on T cell development and ultimately prevents the formation of the peripheral immune system (8–10). Accordingly, understanding the survival mechanisms that operate at different stages of thymocyte development is one key to understanding the complex process of thymopoiesis.

In early thymocyte progenitors, binding of IL-7 and stem cell factor to their respective receptors mediates survival (11–13). However, once a functional pre-TCR is expressed on the surface of the cells at the DN3 stage, this takes over from the cytokines to generate the survival signals that allow pre-T cells to differentiate and proliferate to the CD4/8 DP stage (4, 14; for review see reference 13). The synergistic and overlapping roles of pre-TCR and cytokine signaling in controlling cell survival and differentiation at the DN3 stage has been demonstrated genetically: mice deficient for the recombinase-activating genes (RAG), which are unable to rearrange TCR β subunits, are unable to progress beyond DN3 due to lack of a functional pre-TCR; DN3 cells accumulate in the thymi of these animals (15). In the absence of the common cytokine receptor gamma chain (γc), which causes loss of IL-7 signaling, DN3 cells are lost and only the DN2 population remains (16).

One critical role for IL-7/γc cytokines in the prevention of cell death in $CD25^+$ pre-T cells is controlling the expression of the antiapoptotic protein Bcl-2 (17). The signaling molecules used by the pre-TCR complex to control survival are less characterized, although it is known that the pre-TCR survival checkpoint in DN3 cells is modulated by the tumor suppressor p53. SCID mice which, similar to RAG-deficient mice, are unable to rearrange TCR β subunits, are unable to progress beyond DN3 due to lack of a functional pre-TCR (18). In the absence of p53, both SCID (19–21) and RAG (22) null cells are able to generate CD4/8 DP thymocytes in the absence of TCR β selection. Similarly, mice defective in expression of the CD3γ subunit, an important structural and signaling component of the pre-TCR, show a severe block at DN3 due to increased apoptosis, but crossing onto a $p53^{-/-}$ background strikingly alleviates this block (23). Accordingly, a model has been proposed in which p53 acts as a sensor for correct β selection. Cells can only move forward from the DN3 stage when p53 is removed genetically or inactivated physiologically. Signaling through the pre-TCR complex, a process absolutely dependent on successful rearrangements of the TCR β locus, is crucial to prevent p53-mediated apoptosis.

Previously, we examined the role of the guanine nucleotide binding protein Rho in the thymus (24–26). The bacterial enzyme, C3 transferase from *Clostridium botulinum*, selectively ADP-ribosylates Rho within its effector-binding domain and abolishes its biological function. By using the proximal p56lck promoter to drive thymocyte-specific expression of C3 transferase, it was possible to produce transgenic mice in which the first thymocyte progenitors and subsequent subsets were devoid of Rho function. Rho inactivation severely impairs the generation of normal numbers of thymocytes and peripheral T cells in lck-C3–transgenic mice (24, 26). The most striking problem in lck-C3–transgenic mice is that early $CD25^+$ thymocyte progenitors undergo massive apoptosis at the DN2 and DN3 stage, which leads to severe depletion of these and subsequent thymic subpopulations. There is a second defect in the lck-C3 mice: the few DN4 cells that do develop show decreased rates of cell cycle progression that contribute to the failed expansion and development of CD4/8 DP and SP thymocytes.

As discussed, the DN2 and DN3 cells that die without Rho function are normally dependent on their survival by overlapping signals generated by the IL-7 receptor and the pre-TCR. It is not known whether cells lacking Rho function have defects in the ability of the pre-TCR to control cell survival, particularly the p53 checkpoint, or the ability of IL-7 to control cell survival by regulating Bcl-2 expression. In this context, we previously described that the loss of cellularity caused by loss of Rho function in the thymus can be restored by ectopic expression of a Bcl-2 transgene (24). There is also a striking rescue of DN2 and DN3 thymocyte progenitors by the ectopic expression of Bcl-2. One simple interpretation of this result is that cell death in the Rho-deficient $CD25^+$ cells is caused by failure in IL-7 receptor signaling to upregulate normal levels of Bcl-2. The Bcl-2 experiments in the Rho-deficient thymi provide strong evidence that the defect caused by loss of Rho function is a survival defect but it does not mean a priori that the original cellular defect was loss of Bcl-2. Moreover, the Bcl-2 experiments cannot diagnose whether Rho is regulating death by controlling the p53 checkpoint in pre-T cells. The relationship between Bcl-2 and p53 in thymus survival is indeed complex. Overexpression of Bcl-2 has been demonstrated to protect DP thymocytes against p53-mediated apoptosis induced by DNA damage (27). However, Bcl-2 expression is not sufficient to prevent cell death in pre-TCR–deficient T cell precursors (23, 28).

The object of this study was to explore genetically the possibility of a link between Rho and p53 in the control of pre-T cell survival. We now show that the apoptosis defects caused by loss of Rho function in $CD25^+$ cells are alleviated on a $p53^{-/-}$ background. That is, the loss of Rho function only causes apoptosis in early thymocyte progenitors in the presence of p53. Our results thus identify the GTPase Rho as a regulator of the p53 survival checkpoint during thymopoiesis. We also show that Rho has a role in DP thymocyte survival, but in contrast to the pre-T cell population, this role is independent of p53.

Materials and Methods

Mice. Mice were bred and maintained under specific pathogen–free conditions in the Imperial Cancer Research Fund Biological Resources Unit. Transgenic mice expressing the *C3 botulinum* C3 transferase gene in the thymus under the control of the p56lck promoter were generated as previously described (24, 26). Mice homozygous null for the p53 gene were provided by A. Bradley (Howard Hughes Medical Institute, Baylor College, Houston, TX). Eμ-Bcl-2 mice (strain Bcl-2-25), expressing hu-

man Bcl-2 in the thymus and peripheral T cell compartments, were a gift from S. Cory (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). Litters were typed for inheritance of the transgenes/$p53^{-/-}$ mutation by PCR of genomic DNA.

PCR. Transgene-carrying mice were identified by PCR using transgene-specific primers: C3, G9112 (GCCACCATGGAGCAGAAGCTGATCTCCGG) and C3NT (CTGATTTGCTTAGTCCATAC) or MO142 (GCGCTTACCTGTAGGCATTGC) and C3CT (GGGCACAGCTATCAATCC); Bcl-2, Bcl-2-1 (GGAACTGATGAATGGGAGCAGTGG) and Bcl-2-2 (GCAGACACTCTATGCCTGTGTGG); and p53, 5NTL (GTGGGAGGGACAAAAGTTCGAGGCC) and 3WTL (ATGGGAGGCTGCCAGTCCTAACCC) and F8NEO (TCTCCTGTCATCTCACCTTGC). Genomic DNA was purified from mouse ear punches (or tail snips for p53) and used as template for 35 cycles of PCR (57°C annealing, 72°C elongation, 95°C denaturation).

Cell Preparation. Thymi were obtained by dissection from 4–6-wk-old mice. Tissue was disaggregated by mincing with fine forceps and forced through a fine mesh filter to obtain a single-cell suspension. Total cell numbers were determined by microscopic observation using a Neubauer hemocytometer.

Flow Cytometric Analysis. Antibodies (PharMingen) were obtained conjugated to FITC, PE, or biotin. Biotinylated antibodies were revealed using streptavidin–TRI-COLOR (Caltag Labs.) or streptavidin–allophycocyanin (Molecular Probes). Thymocytes were stained for surface expression of CD8 (53-5.8), CD4 (RM4-5), CD25 (7D4), CD44 (IM7), B220 (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB6-8C5), CD3γ (145-2C11), α/β TCR (GL-3), pan-NK (DX5), anti–mouse Bcl-2 (PharMingen), and Thy1.2 (53-2.1). Cells were stained with saturating concentrations of antibody at 4°C for 20 min at 2×10^6 cells per sample in a 96-well V-bottomed microtiter well plate in 100 μl of PBS containing 1% BSA. Cells were washed with this buffer between incubations and before analysis on a FACSCalibur™ (Becton Dickinson). Events were collected and stored ungated in list mode using CELLQuest™ software (Becton Dickinson). Live cells were gated according to their forward and side scatter profiles, and data were analyzed using CELLQuest™ software.

Intracellular Staining. Thymocytes were isolated and surface receptors were stained as described above. Cells were fixed in 1% paraformaldehyde in PBS for 10 min at room temperature, washed in PBS, and permeabilized with 0.3% saponin permeabilization buffer (0.3% vol/wt saponin, 5% FCS, and 10 mM Hepes, pH 7.4, in PBS) for 10 min at room temperature. Permeabilized cells were then washed, incubated with an anti-FcγRII blocking mAb, and stained with an antibody to the intracellular protein of interest. All washes after permeabilization were performed in saponin wash buffer (0.1% vol/wt saponin, 5% FCS, and 10 mM Hepes, pH 7.4, in PBS).

Apoptosis Assay. Apoptosis within thymic subpopulations was assessed using two protocols: exclusion of 7-amino actinomycin D (7AAD) and plasma membrane staining using annexin V (29). For 7AAD analysis, thymocytes were stained with the appropriate fluorochrome-labeled antibody in DMEM/25 mM Hepes supplemented with 10% FCS. After washing, cells were incubated with 7AAD (Calbiochem-Novabiochem) for 20 min at 4°C in the dark. Cells were analyzed by flow cytometry, with apoptotic cells being identified by 7AAD staining as described (30). For the annexin V staining, thymocytes were stained with the appropriate fluorochrome-labeled annexin V buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) in conjunction with 2.5 mg/ml annexin V-bio (PharMingen), washed, and revealed with streptavidin–TRI-COLOR. For the analysis of thymocyte apoptosis over extended periods of time, cells were cultured in RPMI, 10% FCS, and 5×10^{-5} M 2-ME at 5% CO_2 before staining.

Results

Rho-mediated pre-T Cell Thymocyte Survival Is Controlled by p53. C3-mediated ablation of Rho function in lck-C3–transgenic mice leads to increased levels of apoptosis in $CD25^+$ T cell progenitors, resulting in their elimination and a corresponding depletion of all subsequent developmental stages in the thymus (24). To investigate if cell death caused by the loss of Rho function in pre-T cells was mediated by p53, we examined the consequences of p53 deletion on survival of Rho-deficient thymocytes. Accordingly, p53 knockout mice were crossed with the lck-C3–transgenic mice, and the p53 heterozygous offspring were backcrossed to produce mice expressing the C3 transgene on a $p53^{-/-}$ background. Thymocytes from the different mice were then analyzed for the presence of the $CD25^+$ pre-T cell subset. The data in Fig. 1 A show CD44 and CD25 immunofluorescence analysis of thymocyte progenitors within the CD4/8 DN subset of thymocytes. $p53^{-/-}$ thymocytes have a normal distribution of the DN1-4 T cell progenitors, whereas the depletion of $CD25^+$ cells in the Rho-deficient thymi is readily demonstrated. Strikingly, $CD25^+$ thymocytes were present in the lck-C3/$p53^{-/-}$ double transgenics. Moreover, numerical analysis confirmed the rescue of the $CD25^+$ pre-T cell population in lck-C3/$p53^{-/-}$ double transgenics (Fig. 1 B). The loss of p53 thus prevents the apoptosis induced by loss of Rho function and rescues $CD44^-25^+$ pre-T cells. Although the $CD25^+$ population is restored by the loss of p53 in the lck-C3 transgenics, the CD44/25 staining profiles in Fig. 1 show that these cells fail to accumulate normal levels of DN4 cells. This reflects that loss of Rho function partially inhibits and delays cell cycle progression in DN4 cells, thereby preventing their expansion to normal levels (24).

The proliferation of DN3 and DN4 cells is dependent on the successful rearrangement and expression of TCR β chains. We therefore wished to exclude the possibility that loss of p53 in the Rho-deficient thymi was allowing survival of non–β-selected pre-T cells. Intracellular staining of DN3 cells with an antibody reactive to a common β chain epitope (31) quantitates levels of intracellular β subunits and thus monitors successful β chain rearrangements. The results show the presence of normal levels of intracellular β chains in $CD44^-25^+$ cells from lck-C3/$p53^{-/-}$ mice, indicating that they have apparently undergone normal β selection (Fig. 2).

Rho Is Not Required for Bcl-2 Expression in pre-T Cells. The expression of the survival protein Bcl-2 is developmentally regulated in the thymus (17, 32) and normally upregulated in $CD25^+$ pre-T cells as a consequence of signaling by the IL-7 receptor (17, 28, 33). Our previous observations that ectopic Bcl-2 could prevent apoptosis in Rho-deficient $CD25^+$ cells prompted speculation that Rho

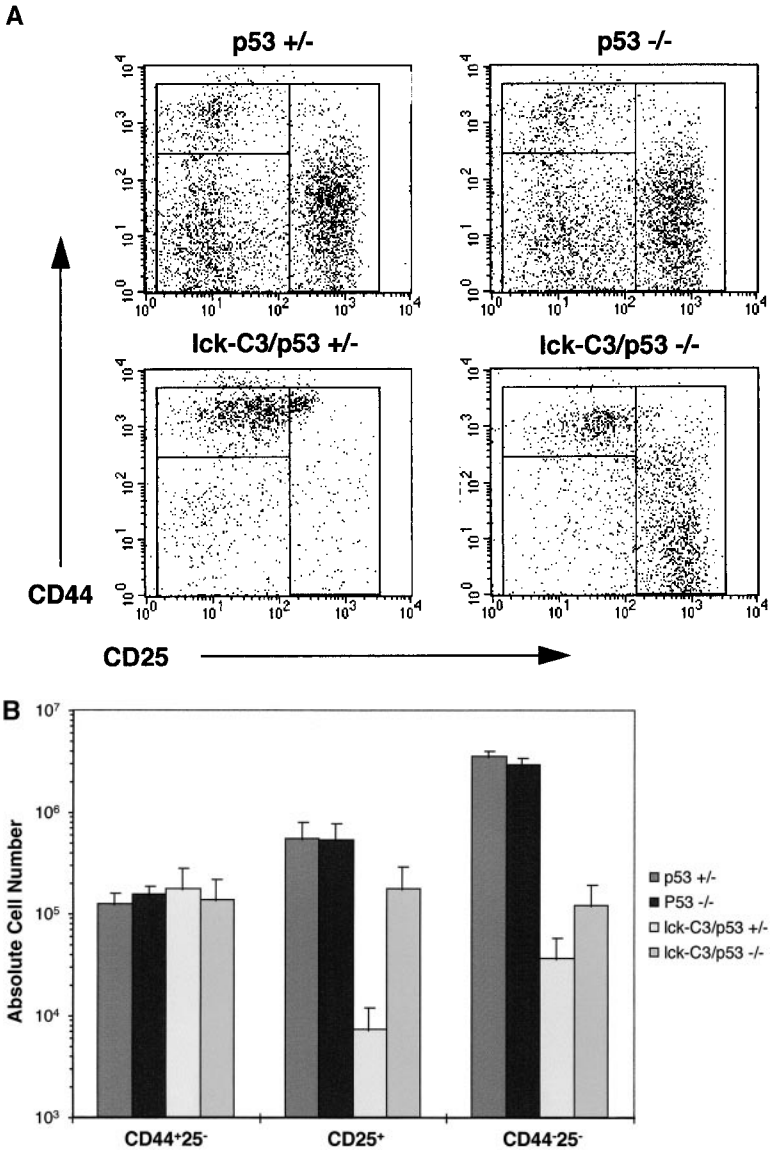

Figure 1. (A) Analysis of CD4/8 DN thymocyte populations in lck-C3/p53$^{-/-}$ mice. Thymi were removed and thymocytes were isolated from p53$^{+/-}$, p53$^{-/-}$, lck-C3/p53$^{+/-}$, and lck-C3/p53$^{-/-}$ transgenic mice and analyzed for expression of CD25 and CD44 by lineage exclusion of all CD4/8 DP and SP thymocytes as well as all cells of non–T cell lineage using a panel of biotinylated antibodies (CD4bio, CD8bio, CD3bio, B220bio, Mac-1bio, NKbio, γδ-bio, and Gr-1bio) revealed with streptavidin–TRI-COLOR, and costained with anti-CD44–PE, anti-CD25–FITC, and Thy1–allophycocyanin. (B) Cell number of CD25/44 thymic populations in lck-C3/p53$^{-/-}$ mice. Absolute cell numbers of CD44^{+}25^{-}, CD25^{+}, and CD44^{-}25^{-} were calculated from p53$^{+/-}$, p53$^{-/-}$, lck-C3/p53$^{+/-}$, and lck-C3/p53$^{-/-}$ transgenic mice. Thy1.2 was used to eliminate non-T cells from the numerical analysis.

might be a critical component of pathways that link the IL-7 receptor to the control of Bcl-2 expression. Indeed, it has been shown in transformed T cell lines that Rho signaling pathways can regulate intracellular levels of Bcl-2 (34). Previously, we did not attempt to examine Bcl-2 levels in Rho-deficient CD25^{+} cells because they are apoptotic, and hence changes in the expression of Bcl-2 might occur as a consequence of apoptosis rather than its cause. However, in lck-C3/p53$^{-/-}$ mice, we prevent the induction of apoptosis in the CD25^{+} population so that sufficient numbers of viable cells are available for examination. Fig. 3 shows immunofluorescence analysis of intracellular Bcl-2 levels in permeabilized DN thymocytes. The data show the pattern of Bcl-2 upregulation at the different stages of early thymocyte development and reveal that intracellular levels of Bcl-2 in pre-T cells are indistinguishable among wild-type, p53$^{-/-}$, and lck-C3/p53$^{-/-}$ mice. These results demonstrate that Rho function is not required for Bcl-2 expression in the thymus.

Loss of Rho Function Results in Increased Apoptosis in CD4/8 DPs by p53-independent Pathways. This study shows that the cell death initiated by loss of Rho function in pre-T cells can be prevented by the removal of p53. However, despite this rescue within the pre-T cell compartment, examination of the gross morphology of the thymus and of total thymocyte numbers revealed that the absence of p53 did not restore cellularity in lck-C3 transgenics (Table I). This result is different from the results seen when ectopic Bcl-2 is used to prevent cell death in lck-C3–transgenic mice. These results were published previously and revealed that overexpression of Bcl-2 in lck-C3–transgenic mice restored the pre-T cell population to normal levels but also partially rescued total thymic cellularity (24). One explanation for this discrepancy is that Rho is required for survival

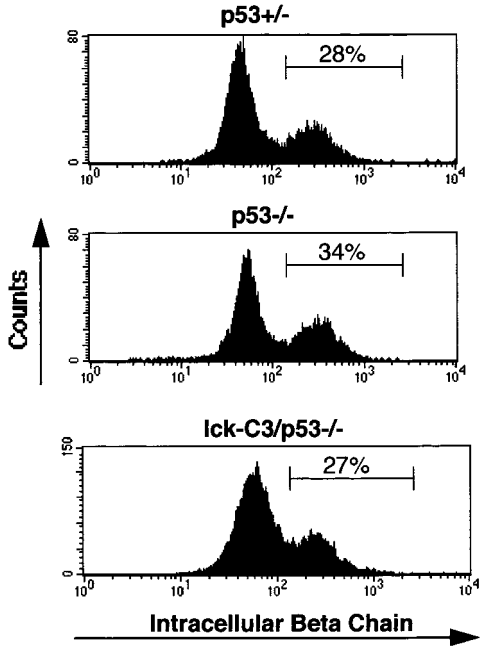

Figure 2. Intracellular β chain staining of $CD25^+$ thymocytes from lck-C3/$p53^{-/-}$mice. Thymocytes from $p53^{+/-}$, $p53^{-/-}$, and lck-C3/$p53^{-/-}$ transgenic mice were stained with anti-CD25–PE and a panel of biotinylated antibodies (CD4bio, CD8bio, CD3bio, B220bio, Mac-1bio, NKbio, γδ-bio, and Gr-1bio) revealed with streptavidin–TRI-COLOR before saponin permeabilization and staining with an antibody to the common β chain epitope.

of thymocytes at later stages of development and that these later pathways for thymocyte survival are p53 independent but can be modulated by Bcl-2. In this context, not only are the lck-C3 thymi extremely small but they also have a very abnormal ratio of CD4/8 DPs and SPs. Typically, DPs should represent 80% of total thymocytes, but in lck-C3 thymi, they approximated 50% of the population. SPs in a normal mouse usually represent 15% of normal thymocytes, whereas in lck-C3 thymi, they were frequently 30–40% of the total population. It should be remembered that total numbers of thymocytes in lck-C3 thymi are severely reduced, so the skewing of DP to SP percentages does not mean that there are more SPs in the lck-C3 thymi. However, the skewing of the ratios of DPs/SPs indicated that there might be selective depletion of DPs in the lck-C3–transgenic mice. We have never before looked at the effects of Rho inhibition on the survival characteristics of DPs. The data in Fig. 4, A and B, address this point. Cells were analyzed by flow cytometry, with apoptotic cells being identified by 7AAD staining (30) and with annexin V (29). The results revealed an increase in apoptotic cells in the CD4/8 DPs isolated from lck-C3–transgenic mice compared with wild-type controls. The data in Fig. 4 D show that the loss of p53 did not prevent apoptosis in CD4/8 DPs from lck-C3–transgenic mice, which was in marked contrast to the protective effect of p53 removal on the cell death caused by loss of Rho function in DN cells (Fig. 4 C). Strikingly, the increased rate of cell death in DPs is reduced to wild-type levels in the lck-C3/Bcl-2 double transgenics (Fig. 5 A). We had hypothesized that

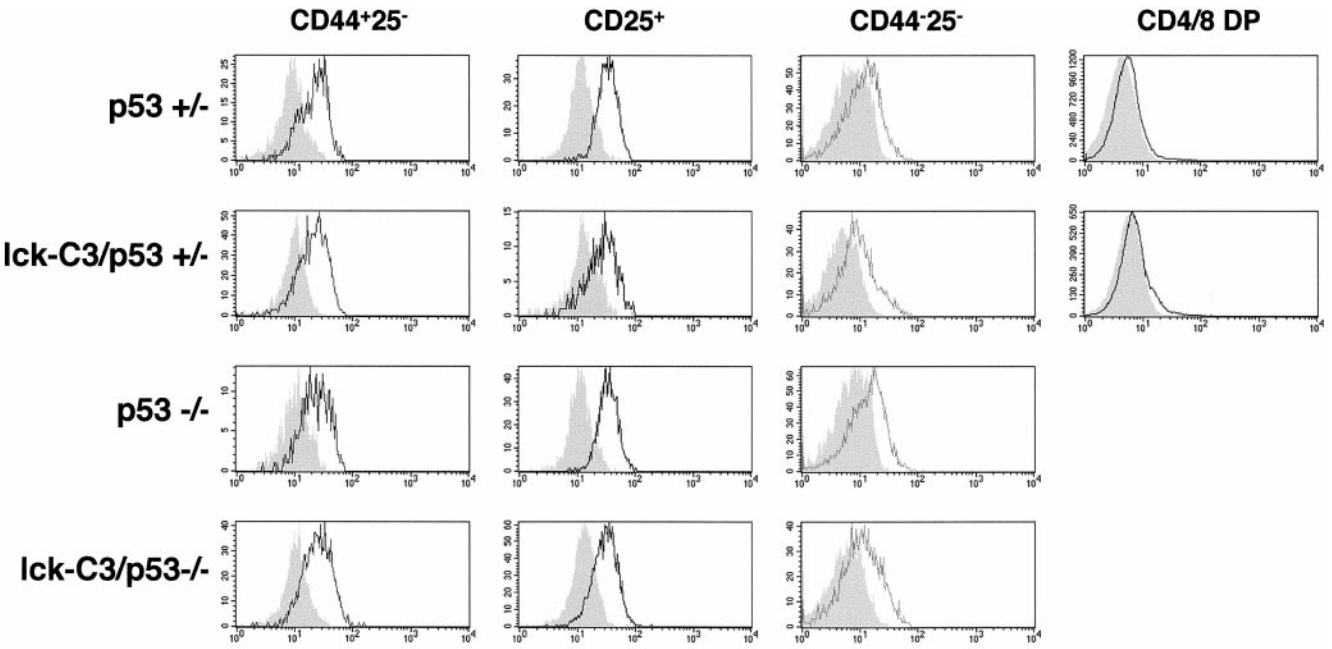

Figure 3. Intracellular Bcl-2 levels in different thymic subsets of lck-C3/$p53^{-/-}$ mice. Thymocytes from $p53^{+/-}$, $p53^{-/-}$, and lck-C3/$p53^{-/-}$ transgenic mice were stained with anti-CD25–PE, CD44–Cy-Chrome, and a panel of biotinylated antibodies (CD4bio, CD8bio, CD3bio, B220bio, Mac-1bio, NKbio, γδ-bio, and Gr-1bio) revealed with streptavidin–allophycocyanin before saponin permeabilization and staining with an antibody to murine Bcl-2 (open histograms). The grey shaded areas represent the staining of an isotype-matched control antibody.

Table I. *Cellularity of lck-C3/p53$^{-/-}$ Double Transgenics*

Mouse	Total thymocytes
	$\times 10^6$
p53$^{+/-}$	196 ± 23
p53$^{-/-}$	202 ± 16
lck-C3/p53$^{+/-}$	9.92 ± 3.3
lck-C3/p53$^{-/-}$	11.9 ± 6.1

Thymi were removed and the number of thymocytes was determined from p53$^{+/-}$, p53$^{-/-}$, p53$^{+/-}$/C3, and p53$^{-/-}$/C3 transgenic mice.

increased cell death was responsible for selective loss of DPs in lck-C3–transgenic mice and hence responsible for the skewed ratio of DP/SP seen in lck-C3 mice (Fig. 5 B). Consistent with this hypothesis, cell death in DPs was prevented by ectopic expression of Bcl-2, and the ratio of DP/SP in the lck-C3 mice was restored to normal (Fig. 5 B). Expression of Bcl-2 also resulted in an increase in the total numbers of DP thymocytes: normally, there are only 4–6 million DPs in lck-C3 mice, compared with 100–150 million DPs in normal littermate controls. Expression of Bcl-2 in the lck-C3 mice restored DPs to 26–34 million. Total numbers of SPs were not changed by Bcl-2 expression.

Discussion

Loss of Rho function in CD25$^+$ pre-T cells results in cell death and the depletion of this population from the thymus. Pre-T cells are dependent for survival on a complex network of overlapping signals generated by cytokines and the pre-TCR. There is also evidence that cell death in T cell precursors involves signaling by 'death' receptors of the TNF receptor (TNFR) family that signal via the adapter Fas-associated death domain protein (FADD)/MORT1 (35). As T cells undergo β chain rearrangements, there is a checkpoint that ensures that only cells that are correctly β selected survive and develop into mature T cells. This checkpoint is monitored by the tumor suppressor p53. This study has explored the role of Rho in controlling p53-mediated apoptosis in the thymus. We have shown previously that when Bcl-2 is ectopically overexpressed, loss of Rho function no longer causes cell death within the pre-T cell population. Cell survival is controlled by a delicate balance between proapoptotic and antiapoptotic signals: overexpression of Bcl-2 pushes the balance in favor of survival (36) but does not mean a priori that the original cellular defect was loss of Bcl-2. Indeed, we show herein that loss of Rho function does not prevent induction of Bcl-2 in pre-T cells. The upregulation of Bcl-2 during early thymocyte development is regulated by IL-7 (17, 28, 33). The presence of normal Bcl-2 levels in thymocytes lacking Rho function is an indication that at least one IL-7–mediated signal for survival operates normally in the absence of Rho. The other main survival pathway in pre-T cells is monitored by p53 (23). Our results demonstrate that Rho controls the cell death response initiated by p53; in the absence of p53, loss of Rho function no longer results in cell death within the pre-T cell population. These data reveal Rho to be a critical component of the signal transduction pathways used to control the proapoptotic p53 checkpoint during TCR β selection.

Previous work has indicated that the pre-TCR complex

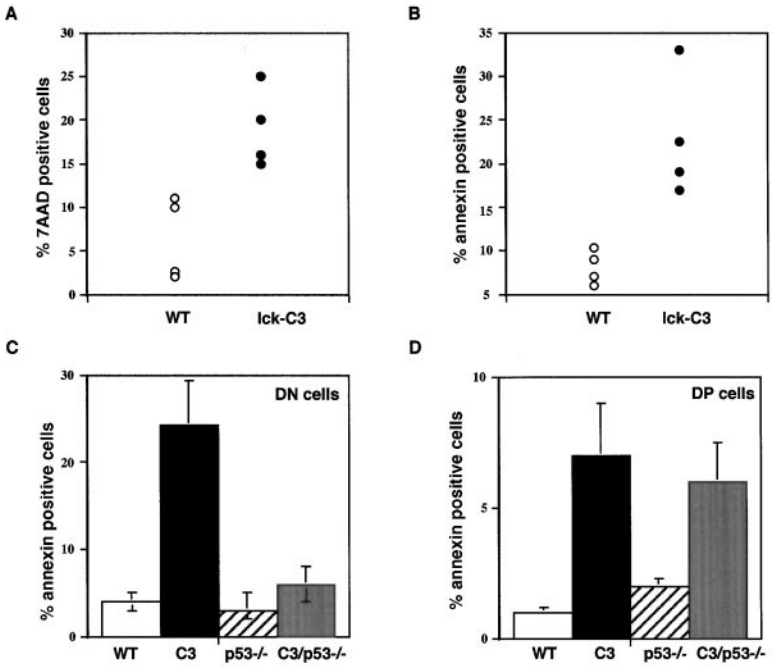

Figure 4. The DN thymic subset is rescued from apoptosis in lck-C3/p53$^{-/-}$ double-transgenic mice. (A) Thymocytes were isolated from 4–6-wk-old mice and placed in culture for 6 h. Cells were stained with CD4–PE, CD8–FITC, and 7AAD to identify apoptotic DP thymocytes. (B) Thymocytes were prepared as in A, stained with CD8–FITC, CD4–PE, Thy1.2–allophycocyanin, and annexin V-bio, which was revealed using streptavidin–TRICOLOR. (C) Rescue from apoptosis of DN thymocytes in lck-C3/p53$^{-/-}$ mice. (D) Failure to rescue DP thymocytes from apoptosis in lck-C3/p53$^{-/-}$ double-transgenic mice. WT, wild type.

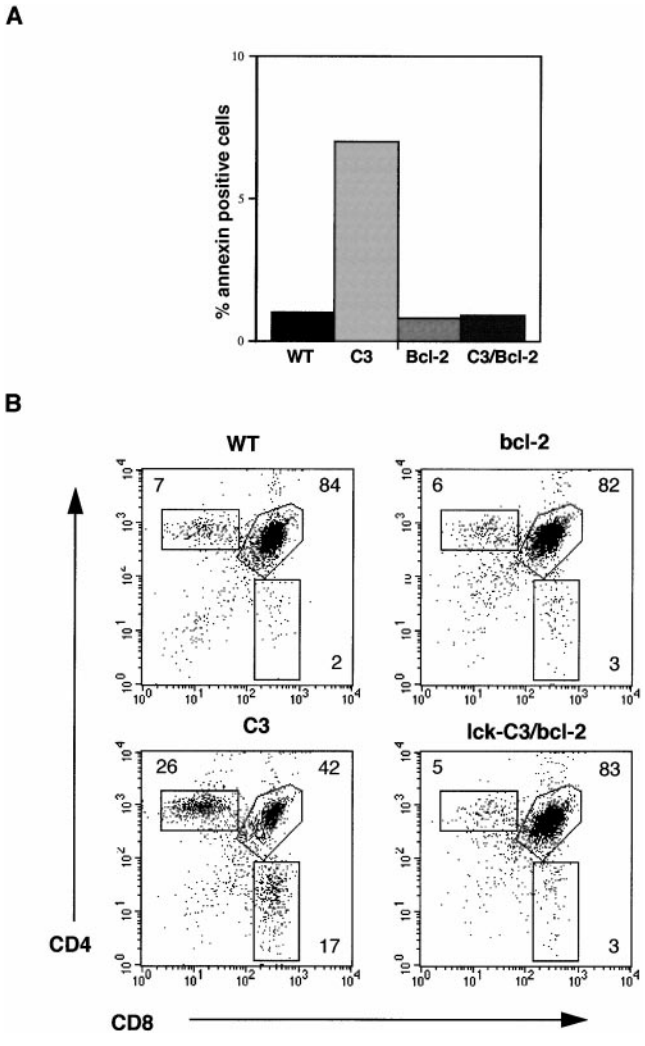

Figure 5. DP thymocytes are rescued from apoptosis in Bcl-2/lck-C3 double-transgenic mice, and the normal SP/DP ratio is restored. (A) Percent of annexin V–positive ex vivo thymocytes staining simultaneously with CD4–PE/CD8–FITC and Th1.2–allophycocyanin. (B) Thy1.2-gated CD4/8 profiles of lck-C3/Bcl-2 double transgenics.

controls p53-dependent cell survival in DN3 cells (23). We now know that Rho also functions to prevent p53-mediated cell death in the thymus. However, we do not think that Rho is in a linear signaling pathway from the pre-TCR complex to counter p53 induction of cell death. We rather view that Rho is part of a parallel system operating in conjunction with the pre-TCR to regulate survival at the DN3 stage. This conclusion is based on a number of experiments that reveal differences between pre-TCR–mediated survival and Rho-mediated survival. First, the survival defect caused by loss of Rho function in DN thymocytes can be rescued by ectopic expression of Bcl-2, whereas pre-TCR–mediated defects cannot. Second, blockade of signaling by death receptors prevents apoptosis in RAG-deficient mice lacking a pre-TCR (35) but not in Rho-deficient thymi (37).

Interestingly, the genetic removal of p53 allowed pre-T cells to survive in the absence of Rho function, but this did not restore thymocyte cellularity. Elimination of p53 is thus not sufficient to compensate for lack of Rho function during all stages of thymocyte development. In contrast, expression of Bcl-2 in Rho-deficient thymi allows survival of pre-T cells and quite significantly reconstitutes total thymic cellularity. The discrepancy between the magnitude of the rescue achieved by Bcl-2 expression versus p53 loss prompted us to search for Rho-regulated, Bcl-2–sensitive but p53-independent survival responses in the thymus. This search resulted in the discovery that Rho function is required for optimum survival of CD4/8 DPs in lck-C3 mice. In this respect, CD4/8 DPs, like pre-T cells, are quite sensitive to p53-mediated apoptosis (38). Nevertheless, loss of p53 had no influence on the level of cell death caused by loss of Rho function in DPs, whereas expression of Bcl-2 was able to alleviate this problem. The picture to emerge from these studies is that Rho is a crucial component of survival signaling pathways in at least two different thymocyte subpopulations: Rho controls the p53 survival checkpoint in pre-T cells and is also crucial for p53-independent survival signaling pathways in CD4/8 DPs. There is one caveat about studies of DPs and SPs in lck-C3 mice, which is that any cell that reaches these developmental stages in the lck-C3 mice has survived the Rho-regulated p53 checkpoint in early progenitors. This could be because the cells compensate for loss of Rho (39), in which case we would underestimate the functional properties of Rho in DPs and SPs.

Finally, this study defines Rho function on the basis of sensitivity to *Clostridium botulinum* C3 transferase. The specificity of C3 transferase for Rho and its failure to target Rac1, Rac2, or Cdc42 is well documented. However, multiple isoforms of Rho exist, and each Rho isoform has the potential to interact with a number of different effectors. A future challenge will be to establish whether the heterogeneity of Rho function in the thymus reflects the expression of different Rho isoforms or different Rho effectors in the various thymocyte subsets.

We wish to thank Ian Rosewell for injection of transgenic constructs and Tracy Grafton, Sam Hoskins, Julie Bee, and Gillian Hutchinson for animal care.

This work was supported by the Imperial Cancer Research Fund (ICRF). P.S. Costello is funded by the European Community Biotech Program (28305). S.C. Cleverley was supported by the ICRF and the Yamanouchi Research Institute. R. Galandrini was supported by the Human Capital Mobility Program (CHRX-CT94-0537), and S.W. Henning was supported by the European Community Training and Mobility of Researchers Program (ERBFMICT 960518).

Submitted: 24 January 2000
Revised: 4 May 2000
Accepted: 5 May 2000

References

1. Godfrey, D.I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol. Today*. 14:547–553.

2. Shortman, K., and L. Wu. 1996. Early T lymphocyte progenitors. *Annu. Rev. Immunol.* 14:29–47.
3. Fehling, H.J., and H. von Boehmer. 1997. Early alpha beta T cell development in the thymus of normal and genetically altered mice. *Curr. Opin. Immunol.* 9:263–275.
4. Dudley, E.C., H.T. Petrie, L.M. Shah, M.J. Owen, and A.C. Hayday. 1994. T cell receptor beta chain gene rearrangement and selection during thymocyte development in adult mice. *Immunity*. 1:83–93.
5. Levelt, C.N., and K. Eichmann. 1995. Receptors and signals in early thymic selection. *Immunity*. 3:667–672.
6. von Boehmer, H. 1992. Thymic selection: a matter of life and death. *Immunol. Today*. 13:454–458.
7. Sebzda, E., S. Mariathasan, T. Ohteki, R. Jones, M.F. Bachmann, and P.S. Ohashi. 1999. Selection of the T cell repertoire. *Annu. Rev. Immunol*. 17:829–874.
8. Von Freeden-Jeffry, U., P. Vieira, L.A. Lucian, T. McNeil, S.E. Burdach, and R. Murray. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J. Exp. Med*. 181:1519–1526.
9. DiSanto, J.P., and H.R. Rodewald. 1998. In vivo roles of receptor tyrosine kinases and cytokine receptors in early thymocyte development. *Curr. Opin. Immunol*. 10:196–207.
10. Cao, X.Q., E.W. Shores, J. Huli, M.R. Anver, B.L. Kelsall, S.M. Russell, J. Drago, M. Noguchi, A. Grinberg, E.T. Bloom, et al. 1995. Defective lymphoid development in mice lacking expression of the common cytokine receptor-γ chain. *Immunity*. 2:223–238.
11. Moore, T.A., and A. Zlotnik. 1995. T-cell lineage commitment and cytokine responses of thymic progenitors. *Blood*. 86:1850–1860.
12. Di Santo, J.P., and H.R. Rodewald. 1998. In vivo roles of receptor tyrosine kinases and cytokine receptors in early thymocyte development. *Curr. Opin. Immunol*. 10:196–207.
13. Haks, M.C., M.A. Oosterwegel, B. Blom, H.M. Spits, and A.M. Kruisbeek. 1999. Cell-fate decisions in early T cell development: regulation by cytokine receptors and the pre-TCR. *Semin. Immunol*. 11:23–37.
14. Hoffman, E.S., L. Passoni, T. Crompton, T.M. Leu, D.G. Schatz, A. Koff, M.J. Owen, and A.C. Hayday. 1996. Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. *Genes Dev*. 10:948–962.
15. Shinkai, Y., G. Rathbun, K.-P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.-M. Stall, et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. 68:855–867.
16. Di Santo, J.P., I. Aifantis, E. Rosmaraki, C. Garcia, J. Feinberg, H.J. Fehling, A. Fischer, H. von Boehmer, and B. Rocha. 1999. The common cytokine receptor gamma chain and the pre-T cell receptor provide independent but critically overlapping signals in early alpha/beta T cell development. *J. Exp. Med*. 189:563–574.
17. von Freeden-Jeffry, U., N. Solvason, M. Howard, and R. Murray. 1997. The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. *Immunity*. 7:147–154.
18. Rothenberg, E.-V., D. Chen, and R.-A. Diamond. 1993. Functional and phenotypic analysis of thymocytes in SCID mice. Evidence for functional response transition before and after the SCID arrest. *J. Immunol*. 151:3530–3536.
19. Bogue, M.A., C. Zhu, E. Aguilar-Cordova, L.A. Donehower, and D.B. Roth. 1996. p53 is required for both radiation-induced differentiation and rescue of V(D)J rearrangement in scid mouse thymocytes. *Genes Dev*. 10:553–565.
20. Guidos, C.J., C.J. Williams, I. Grandal, G. Knowles, M.T. Huang, and J.S. Danska. 1996. V(D)J recombination activates a p53-dependent DNA damage checkpoint in scid lymphocyte precursors. *Genes Dev*. 10:2038–2054.
21. Nacht, M., A. Strasser, Y.R. Chan, A.W. Harris, M. Schlissel, R.T. Bronson, and T. Jacks. 1996. Mutations in the p53 and SCID genes cooperate in tumorigenesis. *Genes Dev*. 10: 2055–2066.
22. Jiang, D., M.J. Lenardo, and C. Zuniga-Pflucker. 1996. p53 prevents maturation to the $CD4^+CD8^+$ stage of thymocyte differentiation in the absence of T cell receptor rearrangement. *J. Exp. Med*. 183:1923–1928.
23. Haks, M.C., P. Krimpenfort, J.H. van den Brakel, and A.M. Kruisbeek. 1999. Pre-TCR signaling and inactivation of p53 induces crucial cell survival pathways in pre-T cells. *Immunity*. 11:91–101.
24. Galandrini, R., S. Henning, and D.A. Cantrell. 1997. Different functions for the GTPase Rho in prothymocytes and late pre-T cells. *Immunity*. 7:163–174.
25. Henning, S.W., and D.A. Cantrell. 1998. p56lck signals for regulating thymocyte development can be distinguished by their dependency on Rho function. *J. Exp. Med*. 188:931–939.
26. Henning, S., R. Galandrini, A. Hall, and D.A. Cantrell. 1997. The GTPase Rho has a critical regulatory role in thymocyte development. *EMBO (Eur. Mol. Biol. Organ.) J.* 16: 2397–2407.
27. Strasser, A., A.W. Harris, T. Jacks, and S. Cory. 1994. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell*. 79:329–339.
28. Maraskovsky, E., L.A. O'Reilly, M. Teepe, L.M. Corcoran, J. Peschon, and A. Strasser. 1997. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor deficient mice but not in mutant $rag\text{-}1^{-/-}$ mice. *Cell*. 89:1011–1019.
29. Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods*. 184:39–51.
30. Schmid, I., C.H. Uittenbogaart, and J.V. Giorgi. 1994. Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. *Cytometry*. 15: 12–20.
31. Buer, J., I. Aifantis, J.P. DiSanto, H.J. Fehling, and H. von Boehmer. 1997. Role of different T cell receptors in the development of pre-T cells. *J. Exp. Med*. 185:1541–1547.
32. Veis, D.J., C.L. Sentman, E.A. Bach, and S.J. Korsmeyer. 1993. Expression of the Bcl-2 protein in murine and human thymocytes and in peripheral T lymphocytes. *J. Immunol*. 151:2546–2554.
33. Kim, K., C.K. Lee, T.J. Sayers, K. Muegge, and S.K. Durum. 1998. The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways. *J. Immunol*. 160:5735–5741.
34. Gomez, J., C. Martinez, M. Giry, A. Garcia, and A. Rebollo. 1997. Rho prevents apoptosis through Bcl-2 expression: implications for interleukin-2 receptor signal transduction. *Eur. J. Immunol*. 27:2793–2799.

35. Newton, K., A.W. Harris, and A. Strasser. 2000. FADD/MORT1 regulates the pre-TCR checkpoint and can function as a tumour suppressor. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:931–941.
36. O'Reilly, L.A., A.W. Harris, and A. Strasser. 1997. bcl-2 transgene expression promotes survival and reduces proliferation of $CD3^{-}CD4^{-}CD8^{-}$ T cell progenitors. *Int. Immunol.* 9:1291–1301.
37. Cleverley, S.C. 1999. Exploring the role of the small GTPase Rho in T lymphocyte biology. Ph.D. thesis. University College London, UK. 145–147.
38. Clarke, A.R., C.A. Purdie, D.J. Harrison, R.G. Morris, C.C. Bird, M.L. Hooper, and A.H. Wyllie. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*. 362:849–852.
39. Cleverley, S.C., S. Henning, and D.A. Cantrell. 1999. Temporal and cell subset specific elimination of Rho function in vivo gives different perspectives on Rho function. *Curr. Biol.* 9:657–660.