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

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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) 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-
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 recombinease-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 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, C9112 (GCCACACATGGAGCATGGTGAG) and C3NT (CTGATTGCTTCTCATTAC) or M0142 (GCCCTTTATTGAGGGTTGACG). Bcl-2, Bcl-2-1 (GGAAGCTGAGATGGGAGCAGTGG) and Bcl-2-2 (GCCACACATGGAGCCTGTTG); and p53, 5NTL (GTGGAGAGGAGCAAAAGTTGAGGCC) and 3WTL (ATGGGAGAGTCGTCCATTAC) and F8NEO (TCTCTCTGTCATCTCACTTTC). 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–TR I-C O L O R (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 FACS Calibur™ (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. Staining 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 Heps, pH 7.4, in PBS) for 10 min at room temperature. Permeabilized cells were then washed, incubated with an anti–FcyRII 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 Heps, 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/N a O H , pH 7.4, 140 mM NaCl, 2.5 mM CaC l 2 ) in conjunction with 2.5 mg/ml annexin V–bio (PharMingen), washed, and revealed with streptavidin–TR I-C O L O R. For the analysis of thymocyte apoptosis over extended periods of time, cells were cultured in RPMI, 10% FCS, and 5 × 10^−5 M 2- M E 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 thymus is readily demonstrated. Strikingly, CD25+ thymocytes were present in the lck-C3/p53−/− double transgens. Moreover, numerical analysis confirmed the rescue of the CD25+ pre-T cell population in lck-C3/p53−/− double transgens (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 transgens, 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 thymus was allowing survival of non-β-selected pre-T cells. Intracellular staining of DN3 cells with an antibody reactive to a common β chain rear1ng 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
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/p53^1/- 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, p53^1/-, and lck-C3/p53^1/- 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 DP s 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.
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 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.

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.
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

| Mouse     | Total thymocytes |
|-----------|------------------|
| p53+/−    | 196 ± 23         |
| p53−/−    | 202 ± 16         |
| lck-C3/p53+/− | 9.92 ± 3.3 |
| lck-C3/p53−/− | 11.9 ± 6.1 |

Table I. Cellularity of lck-C3/p53−/− double transgensics

Thymi were removed and the number of thymocytes was determined from p53+/−, p53−/−, p53+/−/C3, and p53−/−/C3 transgenic mice.
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 thy- 

mocytes 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). Nonetheless, 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.

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