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Baxα perturbs T cell development and affects cell cycle entry of T cells

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Baxα can heterodimerize with Bcl-2 and Bcl-X_L, countering their effects, as well as promoting apoptosis on overexpression. We show that *baxα* transgenic mice have greatly reduced numbers of mature T cells, which results from an impaired positive selection in the thymus. This perturbation in positive selection is accompanied by an increase in the number of cycling thymocytes. Further to this, mature T cells overexpressing Baxα have lower levels of $p27^{Kip1}$ and enter S phase more rapidly in response to interleukin-2 stimulation than do control T cells, while the converse is true of *bcl-2* transgenic T cells. These data indicate that apoptotic regulatory proteins can modulate the level of cell cycle-controlling proteins and thereby directly impact on the cell cycle.

Keywords: apoptosis/Baxα/Bcl-2/cell cycle/T cell selection

Introduction

Apoptosis, or programmed cell death, is a widespread process used to eliminate unwanted or damaged cells from multicellular organisms. For instance, apoptosis serves to ensure the selection of appropriate lymphoid populations during thymic T cell development (Surh and Sprent, 1994). Apoptosis is an active process which is controlled by the expression of certain genes which appear to be highly conserved from nematodes to mammals. Some gene products block apoptosis, whereas others are effectors of apoptosis (reviewed in White, 1996). It may very well be that by understanding the functions of these gene products, a more precise delineation of cell death at the biochemical level can be achieved.

One of these gene products is Baxα, which was isolated by virtue of its interaction with Bcl-2. Alternative splicing within the *bax* gene produces three types of transcript of which the α mRNA encodes the 21 kDa protein that heterodimerizes with Bcl-2 (Oltvai *et al.*, 1993). In this same study, the overexpression of Baxα in an interleukin-3 (IL-3)-dependent cell line is seen to accelerate apoptosis upon removal of the cytokine and relies on the Baxα:Bcl-2 ratio. Thymocytes from mice expressing a *baxα* transgene in the T cell lineage show accelerated cell death in response to dexamethasone and DNA damage (Brady *et al.*, 1996). p53 has been shown to activate transcription of the *bax* gene directly (Miyashita and Reed, 1995). However, we have shown that Baxα overexpression alone is insufficient to restore DNA damage-induced apoptosis to p53(–/–) thymocytes (Brady *et al.*, 1996).

Bcl-2 and its relative Bcl-X_L (Boise *et al.*, 1993) are potent inhibitors of apoptosis induced by various stimuli such as irradiation, glucocorticoid treatment, calcium ionophores or cytotoxic drugs (Sentman *et al.*, 1991; Strasser *et al.*, 1991; Chao *et al.*, 1995). However, unlike its related protein Ced-9 which blocks all programmed cell deaths in *Caenorhabditis elegans* (Vaux *et al.*, 1992; Hengartner and Horvitz, 1994), Bcl-2 does not inhibit all forms of apoptosis in mammalian cells. A *bcl-2* transgene has virtually no effect on the deletion of autoreactive B and T cells (Sentman *et al.*, 1991; Strasser *et al.*, 1991, 1994), nor does it inhibit tumour necrosis factor-α (TNFα)-induced cell death (Vanhaesebroeck *et al.*, 1993) or Fas-mediated apoptosis (Strasser *et al.*, 1995).

It has been suggested that the development and proper function of an effective immune system require apoptosis (Cohen *et al.*, 1992). Immature T cells undergo random rearrangement of their antigen receptor genes (reviewed in Davis, 1990). Only those cells bearing T cell receptors (TCRs) of appropriate specificity are positively selected for survival and further differentiation. The rest, which is at least 95% of the $CD4^+CD8^+$ T cell precursors (Egerton *et al.*, 1990), undergo apoptosis (Surh and Sprent, 1994). Those apoptozing cells are either deleted due to expression of self-reactive receptors (negative selection) or die because they failed to receive a surviving signal (death by neglect) (von Boehmer, 1994). The cellular mechanisms involved in positive or negative selection are not yet outlined fully nor is the role of apoptosis-related molecules, if any, in the process. However, for example, *bcl-2* expression has been shown to be up-regulated during or directly following positive selection (Linette *et al.*, 1994).

Like apoptosis, cell division is a ubiquitous process in multicellular organisms. Unlike apoptosis, much is known about the biochemical signals associated with cell cycle progression and mitosis. Evidence exists to suggest that the two processes may be interconnected (reviewed in King and Cidlowski, 1995; Meikrantz and Schlegel, 1995). For instance, manipulation of the cell cycle can induce apoptosis (Shan and Lee, 1994). Moreover, the transition from G_1 to S phase in mammalian cells is influenced by p53 (reviewed in Cox and Lane, 1995) as well as the activation of cyclin-dependent kinases (CDKs) (reviewed in Morgan, 1995). Both p53 (Yonish-Rouach *et al.*, 1991) and CDK activation (Meikrantz *et al.*, 1994) have also been linked to apoptosis.

We show that Baxα overexpression can lead to a defect in T cell maturation which reflects a perturbation of T

cell selection. It has been shown previously that apoptosis is the major process whereby thymocytes die during selection (Suhr and Sprent, 1994). Our data highlight the fact that apoptotic regulatory molecules such as Baxα can influence T cell selection directly. Our data also show that Baxα is involved in distinct apoptotic pathways and that its action requires protein synthesis. We further demonstrate that Baxα overexpression can facilitate the entry of T cells into S phase whereas Bcl-2 delays this entry via modulation of the level of $p27^{Kip1}$ protein, thus establishing, for the first time, a molecular connection between apoptosis and cell cycle control.

Results

Baxα accelerates apoptosis in T cells and requires protein synthesis for its action

We have shown previously that the T cells from *baxα* transgenic mice show accelerated apoptosis in response to specific stimuli such as dexamethasone or radiation (Brady *et al.*, 1996). To assess the effect of Baxα on survival, thymocytes from *baxα* transgenic mice and control littermates were cultured in medium. Samples were harvested at various time points and the percentage of apoptotic cells determined using a flow cytometry-based technique which measures propidium iodide staining of DNA after cell lysis in a hypotonic buffer (Nicoletti *et al.*, 1991). Over a 3 day period, substantially more apoptosis is seen in the *baxα* thymocytes than in control thymocytes (Figure 1A). By 72 h, 40–50% of control thymocytes still had diploid DNA content, whereas virtually all the *baxα* thymocytes were apoptosed. Similarly, splenic T cells from *baxα* transgenic mice showed accelerated apoptosis compared with splenic T cells from control littermates (data not shown).

It has been suggested that apoptosis in thymocytes is protein synthesis dependent (Sellins and Cohen, 1987) The opposite is true in, for instance, Fas-induced apoptosis of T cells where cycloheximide acts synergistically to accelerate apoptosis (Ogasawara *et al.*, 1995). We sought to determine whether this remained the case in thymocytes from *baxα* transgenic mice. Thymocytes from *baxα* transgenic mice and control littermates were cultured *in vitro* and the level of apoptosis measured after 18 h culture as described above. Baxα-mediated acceleration of thymocyte apoptosis *in vitro*, as seen in Figure 1A, is not evident in the presence of 30 μg/ml cycloheximide (Figure 1B). We further tested whether this was also true when thymocytes were exposed to a specific apoptotic stimulus such as γ-irradiation. Previous work has demonstrated that apoptosis induced by γ-irradiation is diminished in thymocytes in the presence of cycloheximide (Sellins and Cohen, 1987). We now show that even the overexpression of Baxα does not reverse this effect (Figure 1B). This suggests that accelerated apoptosis by Baxα overexpression may require *de novo* synthesis of downstream effector molecules. Such molecules may only be induced in the presence of Baxα plus an apoptotic stimulus or may have a very short half-life. The level of Baxα protein was not diminished noticeably after the incubation with cycloheximide, as judged by Western blot (data not shown).

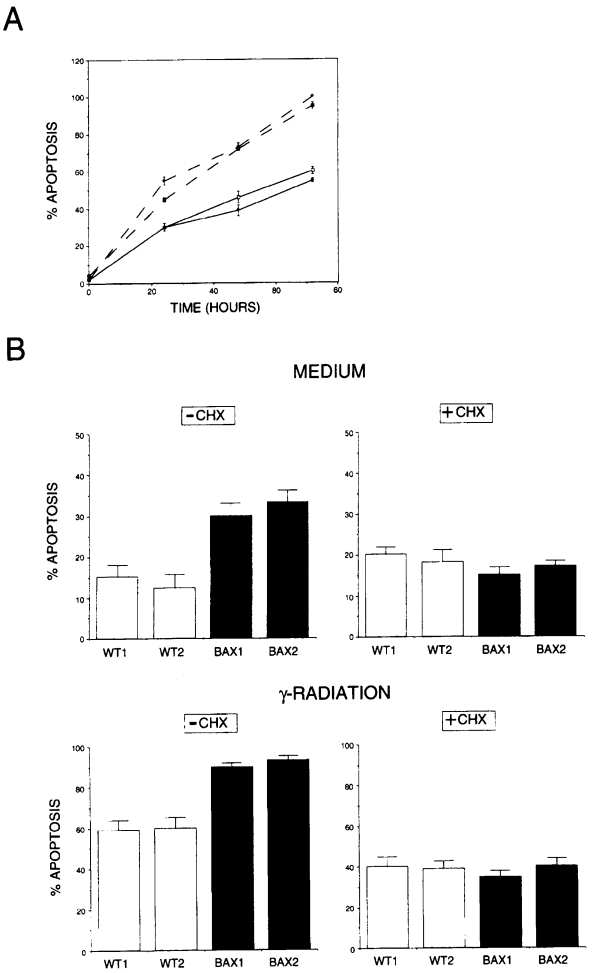


Fig. 1. Baxα accelerates thymocyte apoptosis during culture *in vitro* and requires protein synthesis for its action. (**A**) Thymocytes from *baxα* transgenic mice die faster during *in vitro* culture. Thymocytes were isolated from *baxα* transgenic mice and non-transgenic littermates and cultured in medium at 1×10^6 cells/ml. The percentage of apoptosis of *baxα* transgenic thymocytes (○, ■) is represented by broken lines and control littermate thymocytes (□, ●) by continous lines. Similar results to those shown were observed in four independent experiments involving both the Bax18 and Bax25 lines. Each data point represents the mean ± range of duplicate determination of apoptosis in 10 000 cells each. (**B**) Baxα-accelerated apoptosis requires protein synthesis. Thymocytes from *baxα* transgenics (BAX1, BAX2) and control littermates (WT1, WT2) were cultured in medium for 20 h in the presence or absence of 30 μg/ml of cycloheximide (CHX). The bottom panel shows a similar experiment in which thymocytes were first exposed to 100 cGy of γ-radiation and then incubated for 24 h with or without cycloheximide. Each data point represents the mean ± range of duplicate determinations as described above, and similar results were obtained in four independent experiments.

Baxα acts in distinct apoptotic pathways

Previously, we have shown that Baxα overexpression accelerates apoptosis in thymocytes in response to DNA damage caused by etoposide or γ-irradiation as well as glucocorticoid treatment (Brady *et al.*, 1996). We now have investigated whether this also holds true for other pathways leading to cell death. Fas/APO-1 is a cell surface protein that induces apoptosis in immature thymocytes treated with an anti-Fas antibody (Ogasawara *et al.*, 1995). Fas is a member of the TNF receptor family. TNFα is an apoptosis-inducing cytokine which shares amino acid

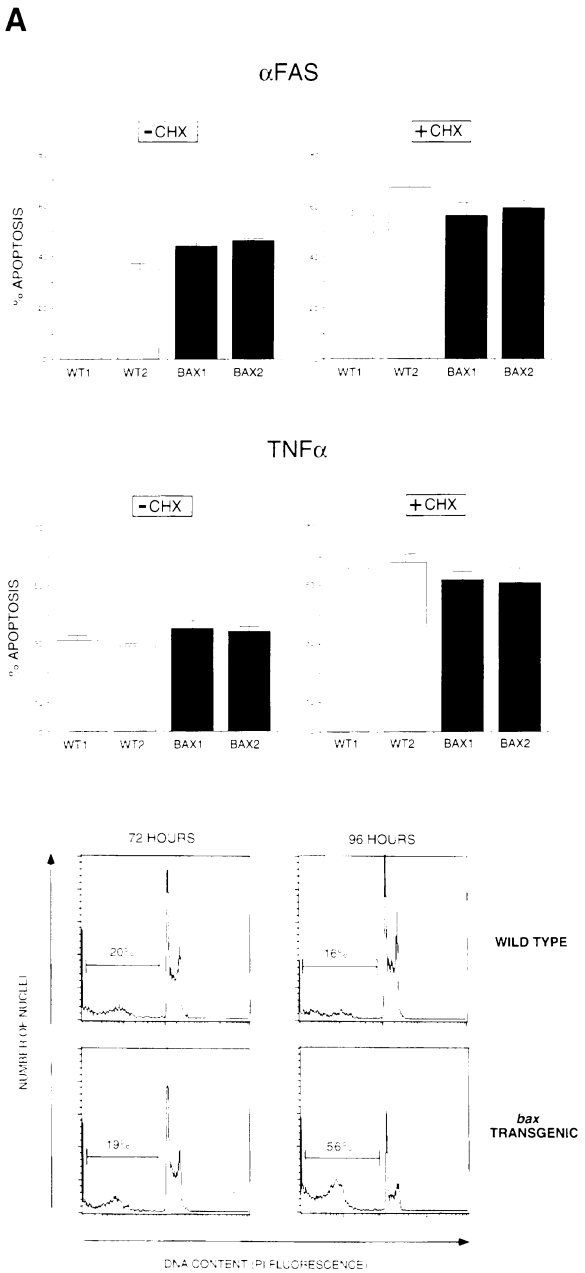


Fig. 2. Baxα does not accelerate apoptosis in T cells in response to Fas and TNFα but does accelerate apoptosis upon IL-2 withdrawal from activated T cells. (**A**) Thymocytes (1×10^6) from *baxα* transgenics (BAX1, BAX2) and control littermates (WT1, WT2) were incubated with 1 μg/ml anti-Fas antibody (Jo2) or 10 ng/ml of recombinant murine TNFα in the presence or absence of 30 μg/ml of cycloheximide for 20 h. Control samples without anti-Fas or TNFα in medium alone had similar values to those shown in Figure 1B. (**B**) Baxα accelerates apoptosis in activated T cells in the absence of exogenous IL-2. Splenocytes from *baxα* transgenics and control littermates were incubated with ConA for 72 h, resulting in a population of virtually pure T cells. Exogenous IL-2 was not added and the percentage of apoptosis was determined at subsequent time points. Similar results were obtained for duplicate samples of 10 000 cells each for duplicate sets of mice tested in four independent experiments.

homology with the ligand for Fas (Smith *et al.*, 1994). We incubated thymocytes from *baxα* transgenic mice and control littermates with 1 μg/ml anti-Fas antibody (Jo2) for 20 h both in the absence and in the presence of 30 μg/ml cycloheximide. Cycloheximide has been shown

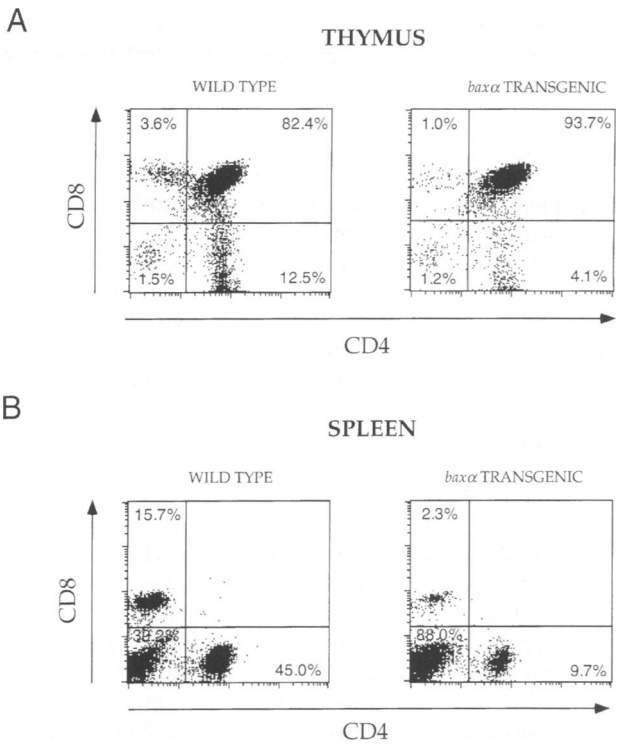


Fig. 3. Baxα transgenics have decreased numbers of mature T cells *in vivo*. CD4 and CD8 expression on thymocytes and splenocytes from *baxα* transgenics and control littermates. The percentage of cells in each population is indicated.

previously to enhance the apoptotic action of anti-Fas antibody on mouse thymocytes (Ogasawara *et al.*, 1995). As on thymocytes from normal mice (Ogasawara *et al.*, 1995), virtually all thymocytes from *baxα* transgenic mice express Fas/APO-1 (data not shown). No accelerated apoptosis was found in *baxα* transgenic thymocytes in response to anti-Fas antibody, either with or without cycloheximide (Figure 2A). A similar result was found when *baxα* and control thymocytes were incubated with 10 ng/ml of TNFα for 20 h. These data show that overexpression of Baxα does not accelerate the cell death pathways initiated in thymocytes by activating Fas with an agonistic antibody or treatment with TNFα.

We also examined the effect of limiting IL-2 on activated T cells. Splenocytes from *baxα* transgenic mice and control littermates were incubated with concanavalin A (ConA) for 72 h, resulting in a virtually pure population of cycling T cell blasts. Incubation for a further 24 h without addition of exogenous IL-2 results in substantial apoptosis (56%, Figure 2B) in T cell blasts from *baxα* transgenic mice but not so much in those from control littermates (16%). The addition of exogenous IL-2 similarly protected both Baxα and control T cell blasts from apoptosis after ConA activation (data not shown). Similar data were obtained after extensive washing of 72 h ConA blast cultures and subsequent incubation in medium without IL-2 (data not shown). Therefore, Baxα overexpression clearly contributes to accelerated cell death in IL-2-dependent activated primary T cells.

Baxα transgenics have decreased numbers of mature T cells in vivo

Having established above that *baxα* transgenic T cells show accelerated cell death *in vitro*, the constitution of T

cell compartments *in vivo* was also scrutinized. Thymocytes from *baxα* transgenic mice and control littermates were analysed by two-colour flow cytometry using antibodies against CD4 and CD8. As shown in Figure 3A, a considerable decrease in the number of $CD4^+$ single positive (SP) and $CD8^+$ SP cells was seen in the *baxα* transgenic mice in comparison with control littermates. The *baxα* transgenic mice have only 25–30% of the control levels of SP cells in the thymus. This was consistent for two independent transgenic lines studied, Bax18 and Bax25 (Brady *et al.*, 1996) and could be observed at all the ages studied, i.e. 2–8 weeks. This change is also reflected in a decrease in the absolute number of SP thymocytes, as the total number of thymocytes did not vary significantly between *baxα* transgenic and control littermates in the age range studied, as reported previously (Brady *et al.*, 1996). Concomitant with the decrease in SP T cells in the *baxα* transgenic thymus is an increase in the number of $CD4^+CD8^+$ double positive (DP) immature thymocytes. A similar analysis was carried out on red cell-depleted splenocytes (Figure 3B). As for thymocytes, a substantial reduction in $CD4^+$ and $CD8^+$ SP T cells was seen in the spleen of *baxα* transgenic mice. These results show that a consequence of the expression of the *baxα* transgene in T cells is a major reduction in the numbers of mature T cells *in vivo*.

T cell selection is perturbed in baxα transgenics

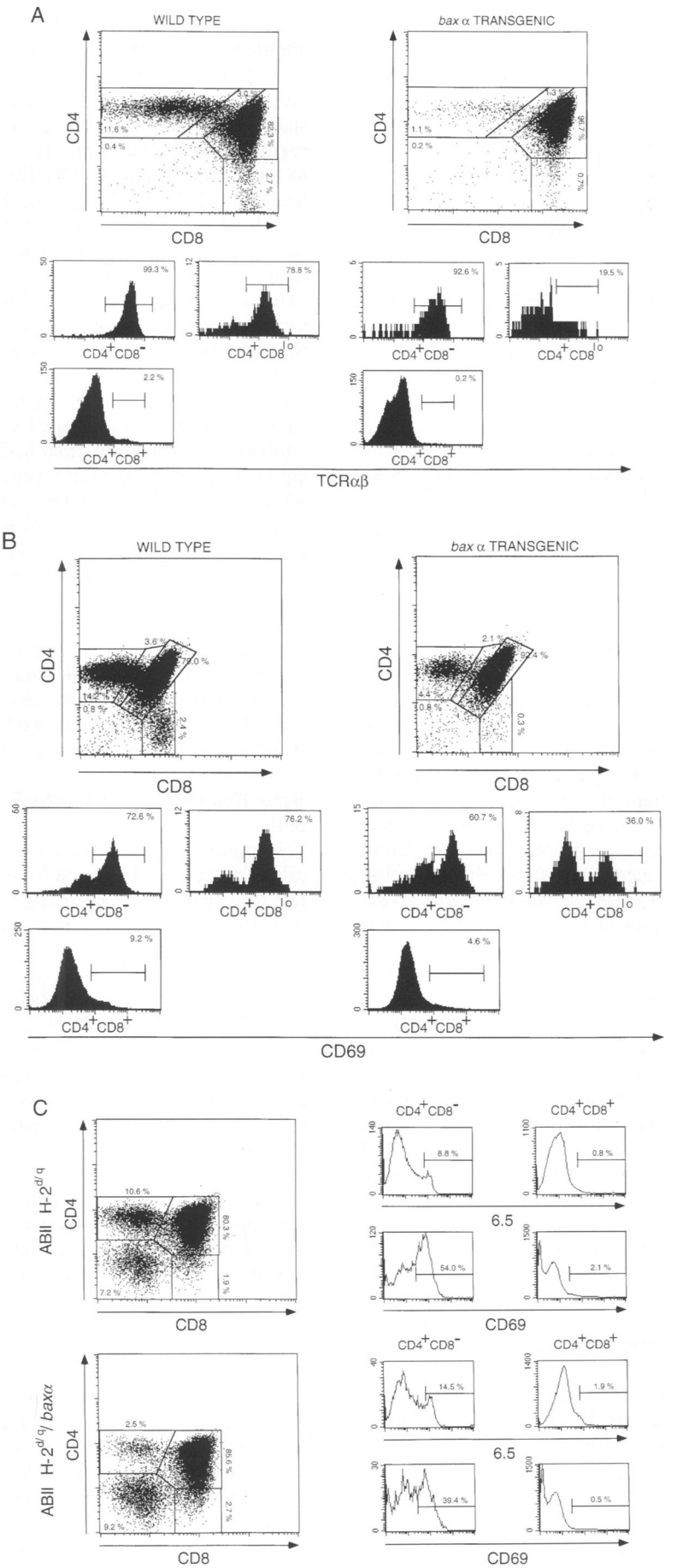
To examine in more detail the defect in T cell development in the *baxα* transgenic mice, we carried out three-colour flow cytometry analysis with antibodies specific for CD4, CD8 and TCR β chain (pan-TCRβ). This allowed us to determine the level of TCRαβ expression on the thymocyte subsets defined on the basis of their CD4/CD8 phenotypes. During positive selection, a small fraction of the immature DP thymocytes will differentiate into SP cells and this is preceded by an up-regulation of the surface expression of TCR in DP thymocytes (reviewed in von Boehmer, 1994). In the wild-type situation, a very small fraction of DP thymocytes show high levels of TCRαβ expression. During differentiation towards $CD4^+$ SP mature T cells, positively selected immature T cells become first $CD4^+CD8^{lo}$ then $CD4^+CD8^-$ (Guidos *et al.*, 1990; Bendelac *et al.*, 1992). The level of TCRαβ expression increases greatly from DP cells to $CD4^+CD8^{lo}$ and increases again, though not so dramatically, in the mature $CD4^+CD8^-$ T cell stage (Figure 4A). However, when the level of TCRαβ expression within the gated subsets of thymocytes from *baxα* transgenic mice is analysed, a different picture is found. The number of $TCR\alpha\beta^{hi}$-expressing cells is decreased greatly in the DP compartment (2.2 to 0.2%) as well as in the transitional $CD4^+CD8^{lo}$ compartment (78.8 to 19.5%). However, the level of TCRαβ expression on the fully mature $CD4^+CD8^-$ T cells is very similar in both *baxα* mice and control littermates. Three-colour analysis cannot distinguish between $CD4^{lo}CD8^+$ thymocytes that are precursors to $CD4^+CD8^+$ cells and $CD4^{lo}CD8^+$ thymocytes committed to become $CD4^-CD8^+$ thymocytes (Guidos *et al.*, 1990). Therefore, we confined our analysis to the $CD4^+CD8^{lo}$ population. Similar results were found using an antibody specific for CD3ε in place of anti-TCRβ (data not shown). Our data are indicative of a Baxα-mediated defect in the process governing thymic positive selection.

To obtain more definitive evidence in favour of defective positive selection having a causative role in the T cell deficiency of the *baxα* transgenics, we examined the expression of CD69 in combination with CD4 and CD8. CD69 is an early activation marker which is up-regulated either during or immediately following positive selection (Bendelac *et al.*, 1992; Swat *et al.*, 1993). As with TCR expression described above, increased CD69 expression appears in DP cells undergoing positive selection and rises dramatically as T cells mature to $CD4^+CD8^{lo}$ and $CD4^+CD8^-$ cells. The percentage of CD69-expressing cells is decreased within the DP and $CD4^+CD8^{lo}$ populations of the *baxα* transgenic mice in comparison with control littermates (Figure 4B). However, similar proportions of CD69-positive cells were evident in the $CD4^+CD8^-$ population of *baxα* and control mice. This is a further indication that the consequence of overexpression of the apoptotic effector molecule Baxα may be to hinder positive selection, thus leading to a deficit in mature T cells. We also examined the effect of Baxα in the thymus of mice expressing a class II MHC ($I\text{-}E^d$) restricted TCR (ABII) (Kirberg *et al.*, 1994) by crossing *baxα* $H\text{-}2^q$ mice with homozygous TCR transgenic $H\text{-}2^d$ mice. Compared with *baxα*-negative littermates, there was a 4-fold reduction in the proportion of $CD4^+$ mature T cells in *baxα* mice (Figure 4C). We noted only a slight increase (1.6-fold) in the proportion of $CD4^+CD8^-$ cells expressing the transgenic TCR (visualized with a clonotype-specific mAb, 6.5). Therefore, expression of Baxα affected the maturation of all cells without a significant contribution due to TCR specificity. In line with this, there was a reduction in the proportion of cells expressing CD69, within both $CD4^+8^+$ and $CD4^+8^-$ cells. There was a slight increase in $CD4^-8^+$ cells; however, it turned out that these cells were predominantly $HSA^+6.5^+$ and therefore represent immature cells that are precursors of $CD4^+8^+$ thymocytes (data not shown).

baxα transgenics have an increased number of cycling cells in the thymus

In light of the reduced numbers of mature T cells in *baxα* transgenic mice described above, together with the unchanged number of thymocytes relative to control littermates, as previously reported (Brady *et al.*, 1996), we examined whether this had implications for the number of cycling cells in the thymus. Initially, we analysed

Fig. 4. T cell selection is perturbed in *baxα* trangenics. The expression of TCRβ receptor chain and CD69 on $CD4^+CD8^+$, $CD4^+CD8^{lo}$ and $CD4^+CD8^-$ thymocytes from *baxα* transgenic and control littermates. Thymocytes were stained with antibodies specific for CD4, CD8 and (**A**) TCRαβ or (**B**) CD69 then analysed by three-colour flow cytometry. In each case, the CD4 versus CD8 expression is shown and the analytical gates used to identify $CD4^+CD8^+$, $CD4^+CD8^{lo}$ and $CD4^+CD8^-$ thymocytes are indicated. Beneath the CD4 versus CD8 dot-plots, the histograms show the level of (A) TCRαβ or (B) CD69 expression on the individual thymocyte subsets. (**C**) Thymocytes from ABII $H\text{-}2^{d/q}$ mice with or without the *baxα* transgene were stained with antibodies for CD4, CD8, CD69 and the ABII clonotype (6.5) and analysed by four-colour flow cytometry. The CD4 versus CD8 expression is shown together with analytical gates used to identify the $CD4^+CD8^-$ and $CD4^+CD8^+$ thymocyte subsets. The histograms show ABII TCR (6.5) and CD69 expression within each subset.


A
WILD TYPE
bax α TRANSGENIC
CD4
CD8
11.6 %
0.4 %
2.7 %
1.1 %
0.2 %
99.3 %
78.8 %
92.6 %
19.5 %
2.2 %
0.2 %
CD4+CD8-
CD4+CD8lo
CD4+CD8+
TCRαβ
B
WILD TYPE
bax α TRANSGENIC
3.6 %
14.2 %
0.8 %
2.4 %
2.1 %
4.4 %
0.8 %
0.3 %
72.6 %
76.2 %
60.7 %
36.0 %
9.2 %
4.6 %
CD69
C
ABII H-2d/q
ABII H-2d/q/ baxα
10.6 %
7.2 %
2.5 %
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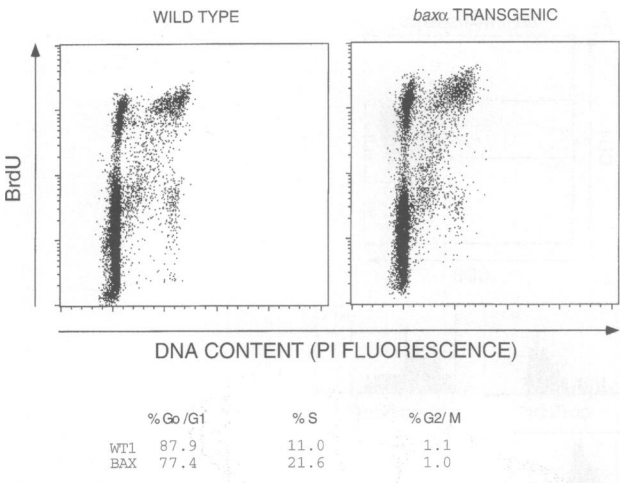

Fig. 5. *baxα* transgenics have an increased number of cells in S phase in the thymus. Mice were injected intraperitoneally with BrdU. The thymus was removed 4 h later, made into a single cell suspension and fixed in 70% ethanol. The fixed thymocytes were stained for BrdU (as a measure of BrdU incorporation) and propidium iodide (PI, as a measure of DNA content) and analysed by flow cytometry. BrdU was plotted against PI fluorescence and the percentage of cells in each phase of the cell cycle was calculated on gating the appropriate population.

thymocytes from *baxα* and control mice for DNA content by propidium iodide staining (Nicoletti *et al.*, 1991). Flow cytometric analysis showed at least a 2-fold increase in the number of cycling cells in *baxα* thymocytes over controls (data not shown). To investigate this further *in vivo* we injected the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) intraperitoneally into *baxα* transgenic mice and control littermates. The thymus was removed from each mouse 4 h later and the BrdU content of the thymocytes measured using a BrdU-specific antibody with propidium iodide counterstaining to assess the DNA content as an indicator of the position in the cell cycle (Begg *et al.*, 1988). We observed that at least twice the number of thymocytes in *baxα* transgenics, compared with control littermates, are in the S phase of the cell cycle (Figure 5). *baxα* thymocytes show a concomitant decrease in the number of cells in G_0/G_1. It could be argued that the increased number of cycling cells is due to the depletion of mature T cells in the *baxα* transgenic mice. The decrease in SP T cells may cause a 'homeostatic stimulus' to compensate for the reduced numbers of mature T cells by increasing the number of immature cells recruited into the cell cycle. This, however, does not address the mechanism whereby this occurs. We sought, therefore, to show whether or not Baxα itself can have a direct effect on the ability of T cells to enter the cell cycle.

Baxα accelerates the entry into S phase of cycling T cells

In order to investigate how Baxα affects S phase entry, we had to look at a situation in which a synchronized population of primary T cells was allowed to re-enter the cell cycle following an exogenous stimulus. To do this, we incubated splenocytes with ConA, as described above, for 72 h. We then starved the resulting T cell blasts of IL-2 for 14 h, which resulted in a completely arrested synchronized population, confirmed by propidium iodide staining and flow cytometry (data not shown). Subsequent addition of IL-2 stimulates the arrested cells to re-enter the cell cycle. Samples were taken at various time points after the addition of IL-2 to allow analysis of cell cycle status as monitored by the kinetics of $p27^{Kip1}$ down-regulation as well as BrdU incorporation. T cells induced to leave a quiescent state by IL-2 show a decrease in the level of $p27^{Kip1}$, allowing CDK2 activation and entry into S phase (Firpo *et al.*, 1994; Nourse *et al.*, 1994). Splenocytes of *baxα* transgenic mice and control littermates were stimulated with ConA for 72 h, washed extensively and starved of IL-2 for 14 h. Upon re-addition of IL-2, samples were taken at various time points and the expression of $p27^{Kip1}$ analysed against time. Equal numbers of viable cells were loaded in each track for immunoblot analysis. The Western blots were probed with a polyclonal antibody specific for $p27^{Kip1}$ and a monoclonal antibody against actin (pan-actin) as a control for the equivalence of the protein levels. As shown in Figure 6A, the level of $p27^{Kip1}$ decreases with time upon addition of IL-2 to the arrested T cell blasts. However, the overall level of $p27^{Kip1}$ is lower in the *baxα* transgenic T cell blasts than in those from control littermates, as illustrated in Figure 6A. To confirm that the lower levels of $p27^{Kip1}$ were reflected in an accelerated entry into S phase, we also analysed the IL-2-re-stimulated blasts for BrdU incorporation. As shown in Figure 6B, the *baxα* transgenic T cell blasts enter S phase faster than T cell blasts from control littermates, as measured by their increased level of BrdU incorporation in the first 18 h after IL-2 stimulation.

Bcl-2 delays the entry into S phase of cycling T cells

The implication of the above data is that overexpression of the apoptotic effector molecule Baxα can influence the rate at which cycling cells enter S phase. To test whether this effect has a wider context than Baxα alone, we tested whether or not Bcl-2 would exert an opposing effect. We generated ConA-stimulated T cell blasts from the spleens of E*μbcl-2* transgenic mice (McDonnell *et al.*, 1989) and control littermates, removed ConA and then starved them for 14 h before adding IL-2. Samples taken at various time points were then analysed for $p27^{Kip1}$ levels and BrdU incorporation. The levels of $p27^{Kip1}$ were higher in the *bcl-2* transgenic T cell blasts, and the rate of decline of these levels was also slower than in controls following IL-2 stimulation (Figure 7A). The CDK inhibitor $p21^{Cip1}$ is induced upon IL-2 stimulation (Nourse *et al.*, 1994). The kinetics of $p21^{Cip1}$ induction for both the *bcl-2* and *baxα* T cell blasts upon IL-2 addition were measured and remained unchanged in comparison with control blasts (data not shown). To verify that the slower decline in $p27^{Kip1}$ levels in the *bcl-2* T cell blasts correlated with a delayed entry into S phase, we measured the level of BrdU incorporation after IL-2 addition. As seen in Figure 7B, the entry of *bcl-2* transgenic T cell blasts into S phase is substantially delayed in comparison with that in control littermates. These data demonstrate that overexpression of a molecule which accelerates apoptosis, such as Baxα, or a molecule that blocks apoptosis, such as Bcl-2, can directly influence the rate at which cells enter the cell cycle: an accelerated rate in the case of Baxα and a delayed rate in the case of Bcl-2.

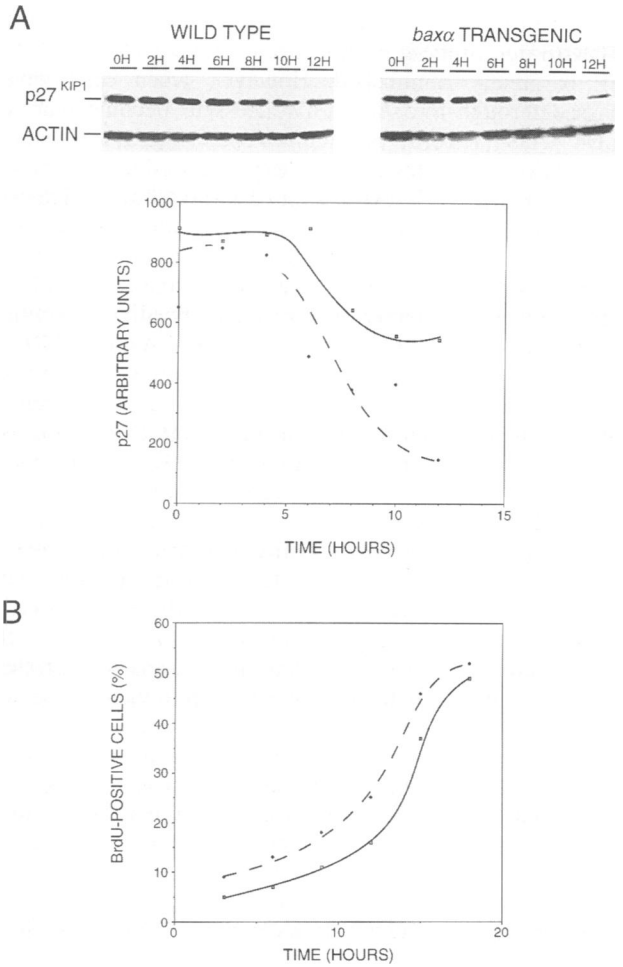

Fig. 6. Bax α accelerates the entry into S phase of cycling T cells. Splenocytes from *baxα* transgenic mice and control littermates were stimulated with ConA for 72 h, washed extensively and starved of IL-2 for 14 h. IL-2 and BrdU were then added to the cultures and samples taken as indicated. (**A**) Expression level of $p27^{Kip1}$ against time. Equal numbers of viable cells (as determined by counting and trypan blue exclusion) were loaded in each track for immunoblot analysis. The Western blots were probed with antibodies specific for $p27^{Kip1}$ and actin (as a loading control) and the level detected by enhanced chemiluminescence and autoradiography. The resulting autoradiograms were scanned by densitometry and the values obtained by dividing the intensity of each $p27^{Kip1}$ band by the corresponding actin band intensity plotted as a function of time following IL-2 readdition. The broken line and closed squares represent the *baxα* transgenic, and the continous line and open squares represent the control littermate. (**B**) Rate of entry into S phase. The rate of entry of the *baxα* and control ConA blasts into S phase following starvation and IL-2 re-addition was calculated by measuring the percentage of BrdU incorporation as in Figure 5. The percentage of BrdU-positive cells (S phase cells) is plotted as a function of time following IL-2 re-addition. Similar data to those shown in (A) and (B) were found consistently in four independent experiments. The broken line and closed squares represent the *baxα* transgenic and the continous line and open squares represent the control littermate.

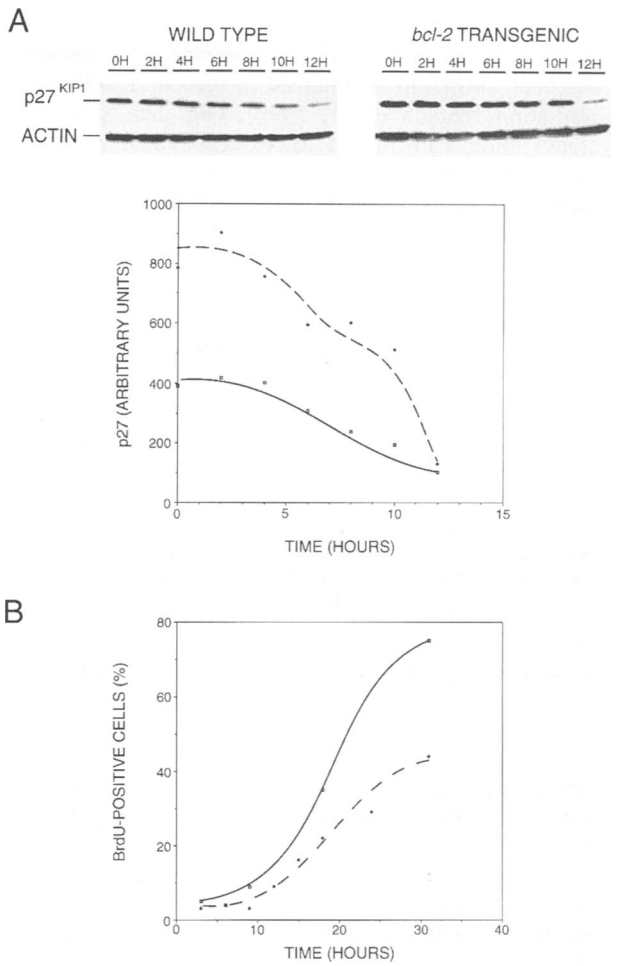

Fig. 7. Bcl-2 delays the entry of cycling cells into S phase. Splenocytes from *bcl-2* transgenic mice and control littermates were stimulated with ConA for 72 h, washed extensively and starved of IL-2 for 14 h. IL-2 and BrdU were then added to the cultures and samples taken as indicated. (**A**) Expression level of $p27^{Kip1}$ against time. Equal number of viable cells, sampled at the time points shown, were probed for $p27^{Kip1}$ and actin as described in Figure 6. The relative levels of $p27^{Kip1}$ (corrected for actin) were plotted as a function of time following IL-2 re-addition. The broken line and closed squares represent the *bcl-2* transgenic and the continous line and open squares represent the control littermate. (**B**) Rate of entry into S phase. The percentage of BrdU-positive cells is plotted as a function of time following IL-2 re-addition. Similar data to those shown in (A) and (B) were found consistently in three independent experiments. The broken line and closed squares represent the *baxα* transgenic and the continous line and open squares represent the control littermate.

Discussion

Baxα overexpression accelerates apoptosis of primary T cells in distinct pathways

Our initial analysis of *baxα* transgenic mice (Brady *et al.*, 1996) showed that both glucocorticoid- and DNA damage-induced apoptosis are accelerated in the presence of Baxα overexpression. In the study presented here, we found that thymocytes and splenic T cells from *baxα* transgenics die faster when cultured *in vitro* without a particular apoptotic stimulus than do control T cells. This is consistent with the antagonistic relationship between Baxα and Bcl-2 (Oltvai *et al.*, 1993) as *bcl-2* transgenic T cells have an extended lifespan in comparison with control cells when cultured *in vitro* (Sentman *et al.*, 1991; Strasser *et al.*, 1991).

Earlier studies have suggested that the induction of DNA fragmentation and hence apoptosis in irradiated or glucocorticoid-treated lymphocytes is dependent on RNA and protein synthesis (Cohen and Duke, 1984; Sellins and Cohen, 1987). One model to explain this is that certain molecules regulating apoptosis need to be induced or processed following the initial apoptotic stimulus in order

to facilitate cell death (Cohen *et al.*, 1992). Baxα is a candidate for being such a molecule since, for instance, it has been shown to be induced *in vivo* in T cells upon γ-irradiation (Kitada *et al.*, 1996). Our data show that cycloheximide can inhibit Baxα-accelerated apoptosis resulting from *in vitro* culture. This suggests that Baxα requires protein synthesis to accelerate apoptosis, which might conceivably entail the induction or activation of downstream cysteine proteases (White, 1996).

Clearly, there are multiple pathways leading to apoptosis which are subject to distinct regulation (Strasser, 1995). We find that the cell death pathway activated via the Fas receptor is not enhanced by overexpression of Baxα. This suggests that signalling through Fas occurs via an alternative pathway to the one involving Baxα. This correlates with the recent finding that overexpression of Bcl-2 in the lymphoid cells of transgenic mice does not inhibit Fas-induced apoptosis (Strasser *et al.*, 1995). Similarly, we find that TNFα-induced apoptosis is not accelerated in Baxα transgenic T cells, which again agrees with earlier data that Bcl-2 is a poor inhibitor of TNF-induced apoptosis (Vanhaesebroeck *et al.*, 1993). It appears that apoptosis triggered via Fas or TNF depends on the interleukin 1β-converting enzyme (ICE) or a close homologue (Enari *et al.*, 1995; Los *et al.*, 1995). It has been suggested that ICE could either act downstream of the point in the apoptosis pathways blocked by Bcl-2 or, alternatively, that ICE and Bcl-2 are in distinct pathways (Strasser, 1995). Our data suggest the latter is more likely, as Baxα overexpression results in accelerated apoptosis in some death pathways but not in others requiring ICE. Baxα does influence apoptosis upon IL-2 withdrawal from cultures of ConA-stimulated T cell blasts. This confirms that the Baxα-accelerated death of an IL-3-dependent cell line upon IL-3 withdrawal (Oltvai *et al.*, 1993) also holds true for primary cells. As a further correlation of the lack of effect in the Fas/TNF pathway, IL-2 withdrawal-induced apoptosis has been shown not to involve ICE or closely related homologues (Vasilakos *et al.*, 1995).

Baxα perturbs T cell development

The Baxα mice have only 25–30% the level of mature SP T cells in thymus and spleen as compared with control littermates, whereas the number of DP thymocytes is slightly increased. This contrasts with the lymphoid hyperplasia reported in *bax* (–/–) mice (Knudson *et al.*, 1995). The instance of a slightly increased number of *baxα* DP thymocytes indicates that until the point of selection there is probably no stimulus to induce apoptosis. When, however, the thymocytes are cultured *in vitro*, the *baxα* DP thymocytes die faster than the control thymocytes (Figure 1A).

The depletion of mature T cells *in vivo* suggests that the *baxα* mice may have a defect in selection, in particular in positive selection. Positive selection is a prerequisite for the development of mature SP $CD4^+$ or $CD8^+$ T cells from their DP precursors (reviewed in von Boehmer, 1994). Before positive selection, DP cells express low levels of TCR/CD3 molecules. One of the first detectable consequences of positive selection is TCR up-regulation (Borgulya *et al.*, 1992). As we show in Figure 4A, the normal up-regulation of TCRαβ expression from the DP stage through the $CD4^+CD8^{lo}$ to the $CD4^+CD8^-$ stage is altered in *baxα* mice. The number of cells up-regulating TCRαβ during the transition from DP via $CD4^+CD8^{lo}$ to SP is greatly diminished. However, when cells have passed through the selection process to become mature $CD4^+CD8^-$ T cells, both controls and *baxα* transgenic mice have similar levels of TCRαβ expression. The same holds true for CD3ε expression (data not shown). This is suggestive of a Baxα-mediated perturbation of positive selection. A further indication for this is the pattern of CD69 expression. CD69 is an activation marker which is up-regulated transiently as a result of positive selection (Bendelac *et al.*, 1992; Swat *et al.*, 1993). As for TCRαβ and CD3ε, fewer cells express high levels of CD69 within the DP and $CD4^+CD8^{lo}$ populations in *baxα* transgenic mice than in controls. However, CD69 expression is virtually identical on the mature $CD4^+CD8^-$ cells of both *baxα* and control mice. Our data are similar to those reported for *vav*-deficient thymocytes which are said to reflect defective positive selection (Fischer *et al.*, 1995). Transgenic mice expressing a dominant-negative version of Nur77, which has a role in TCR-mediated apoptosis, have defective negative selection (Calnan *et al.*, 1995). It is of interest to note that, in contrast to *baxα* mice, these mice show up-regulated CD3 expression in the thymocyte compartments and no change in CD69 levels.

Immature thymocytes that are not positively selected are thought to die via negative selection or neglect (Kersh and Allen, 1996). It has been shown that apoptosis is the major process whereby thymocytes die and that the vast majority of apoptotic cells in the thymus seem to be a reflection of failure to undergo positive selection (Surh and Sprent, 1994). Our data indicate that overexpression of an apoptosis-accelerating molecule, such as Baxα, can cause failure of positive selection directly. As the cellular mechanisms of positive selection are not yet known, it is difficult to speculate how Baxα or other apoptotic effector molecules influence such events. Since selection is a complex and sensitive process dependent on variations in avidity and affinity (Kersh and Allen, 1996), it is possible that the presence of overexpressed Baxα could increase the threshold for survival in such a way as to decrease the number of cells positively selected. The data from the ABII TCR *baxα* mice show no obvious influence of the TCR specificity when studying the effect of Baxα in positive selection. However, we have performed these experiments only in one TCR transgenic line thought to represent a high affinity TCR (Kirberg *et al.*, 1994). It is possible that in TCR transgenic lines with other TCR affinities there could be an enrichment or a decrease of TCR transgene-expressing cells relative to those cells that express TCRs of unknown specificity. Therefore, additional studies will be required to determine whether Baxα actually is involved in setting the window of allowed TCR affinities or whether Baxα simply down-regulates positive selection, either by depleting those cells that can be positively selected or via more specific effects.

Evidence exists to show that Bcl-2 can, to some extent, influence positive selection in the opposite fashion to Baxα. Bcl-2 is up-regulated in DP thymocytes undergoing positive selection (Linette *et al.*, 1994). A *bcl-2* transgene has been reported to enhance the selection of $CD4^-CD8^+$ T cells (Linette *et al.*, 1994; Tao *et al.*, 1994), though this was not evident from another study (Strasser *et al.*, 1994).

It will be of interest to see whether or not *bax* (–/–) mice (Knudson *et al.*, 1995) have enhanced positive selection. It should be remembered that molecules involved in apoptosis have some redundancy (White, 1996) and the studies described here with Baxα could be seen as a possible paradigm for the action of a family of apoptotic effector molecules with overlapping functions.

Baxα and Bcl-2 can alter the entry of cycling T cells into S phase

A further aspect of the thymi of *baxα* transgenic mice which we observed was the doubling of the number of thymocytes in the S phase of the cell cycle (Figure 5). This could be attributed to the influence of a homeostatic process within the thymus whereby the depletion of mature T cells, mediated by the *baxα* transgene, is counteracted by an increased recruitment of cells into the cell cycle within earlier compartments in an attempt to fill up the void. Such a process may exist and result in increased cycling, but we were interested in whether Baxα overexpression itself could influence T cell entry into the cell cycle. To assess this, we stimulated splenocytes with ConA to generate T cell blasts, then removed ConA and incubated the cells in medium without IL-2 for 14 h before re-stimulation by adding IL-2. It has been demonstrated that IL-2 allows CDK activation by causing the elimination of the CDK inhibitor protein $p27^{Kip1}$ (Firpo *et al.*, 1994; Nourse *et al.*, 1994). $p27^{Kip1}$ governs CDK activity during the transition from quiescence to S phase in T lymphocytes. We find that Baxα overexpression correlates with overall decreased levels of $p27^{Kip1}$ after IL-2 stimulation and, furthermore, that this correlates with accelerated entry into S phase as measured by BrdU incorporation and propidium iodide counterstaining (Figure 6). Further confirmation of the finding that apoptosis regulatory molecules can have an influence on the cell cycle comes from our observation of the opposite effect in *bcl-2* transgenic T cells, namely that Bcl-2 delays the down-regulation of $p27^{Kip1}$ after IL-2 stimulation with a concomitant delay in S phase entry (Figure 7). We also observe that the absolute levels of $p27^{Kip1}$ are higher in the *bcl-2* blasts compared with controls. One explanation of why the effect on the cell cycle is more pronounced in the *bcl-2* T cells than the *baxα* cells is that the relative increase in transgenic Bcl-2 levels compared with endogenous Bcl-2 is much greater than for Baxα (Brady *et al.*, 1996 and data not shown).

These data pose the questions of how apoptosis regulatory molecules can influence the cell cycle machinery and with what purpose. The two main possibilities for how the interaction can occur are either directly or indirectly. Several analyses (e.g. Harper *et al.*, 1993; Makela *et al.*, 1994) have already taken place for proteins interacting with members of the cell cycle machinery, but to date no interaction with Bcl-2 family members has been reported. However, such an interaction cannot be excluded. The indirect association could be via proteases downstream of Baxα/Bcl-2 whose activities might modulate components of the cell cycle. Thus, the activation of these downstream proteases could be a necessary event contributing to the process of down-regulation of cell cycle inhibitory proteins like $p27^{Kip1}$ necessary for cell cycle progression. Although the ubiquitin–proteasome system has been shown to be involved in the degradation of $p27^{Kip1}$ (Pagano *et al.*, 1995), other proteolytic pathways cannot be excluded. The fact that *bcl-2*, *bax* and *bcl-x* mRNAs are induced by growth factors (Broome *et al.*, 1995; Miyazaki *et al.*, 1995) suggests that these proteins fulfill a role in normal cell cycle progression.

Several reports have demonstrated the importance of the activation of cyclin A/CDK2 complexes in the decision of the cell to complete the cell cycle and continue proliferating or, alternatively, undergo apoptosis (reviewed in Meikrantz and Schlegel, 1995). Although, our data do not unequivocally position the Baxα/Bcl-2 effect within the cell cycle machinery, we have demonstrated that their overexpression results in modulation of $p27^{Kip1}$ levels. As a consequence of this, Baxα/Bcl-2 levels could influence the timing of the activation of cyclin E/CDK2 and cyclin A/CDK2 complexes. In the case of the Baxα T cell blasts, the facilitated activation of the cyclin/CDK2 complexes would then allow cells to progress more quickly towards a point in the cell cycle where the decision is made either to live or die. At the decision point, Baxα may also have an additional effect to favour apoptosis. The opposite situation would apply in Bcl-2 blasts where the decision point is reached more slowly.

We have shown here the effects of Baxα/Bcl-2 on the cell cycle and T cell selection. These may be separate functions of the same proteins or they could be interconnected. One way to explain both effects is based upon the observation that non-dividing immature T cells, in particular, are positively selected (Huesmann *et al.*, 1991). Baxα overexpression would lead to more T cells in the cycle whereas Bcl-2 would do the reverse.

Our data indicate the importance of apoptosis regulatory molecules in influencing both the course of T cell selection and the cell cycle. Further research will focus on the effects of Baxα/Bcl-2 on cyclin/CDK complexes during the cell cycle as well as on mechanistic aspects of Baxα in positive selection.

Materials and methods

Transgenic mice

The *baxα* transgenic mice used were lines Bax18 and Bax25 which have the same phenotype as described previously (Brady *et al.*, 1996). The ABII H-$2^{d/d}$ TCR transgenic mice used were as described previously (Kirberg *et al.*, 1994). The Eμ*bcl-2* transgenic mice used were a gift of T.McDonnell and S.Korsmeyer (McDonnell *et al.*, 1989). The line was mantained on a C57 BL/6J background then backcrossed once on FVB before being used in the experimental procedures described.

Apoptosis assays

The assays measuring response to apoptosis were performed as previously described (Brady *et al.*, 1996). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 50 μM 2-mercaptoethanol. Flow cytometric quantitation of apoptotic cells was performed as indicated in Nicoletti *et al.* (1991). Anti-Fas antibody (Jo2) was purchased from Pharmingen (San Diego, CA). Recombinant murine TNFα was purchased from Prepro Tech Inc (Canton, MA), and cycloheximide and propidium iodide from Sigma (St Louis, MO).

Flow cytometric analysis

Single cell suspensions were prepared from thymi or spleens. Cells were incubated for 30 min on ice with the appropriate dilution of the corresponding antibodies. Fluorescein isothiocyanate (FITC)-conjugated antibodies to TCRαβ (H57-597), CD3 (145-2C11) and CD8α (53-6.7), phycoerythrin-conjugated antibody to CD4 (RM4-5) and biotinylated antibodies to CD8α (53-6.7) and CD69 (H1.2F3) were purchased

from Pharmingen (San Diego, CA). Anti-mouse CD8a (clone 53-6.7) RED613™ conjugate was from Gibco-BRL. The 6.5 monoclonal antibody (Kirberg *et al.*, 1994) was labelled with fluorescein hydroxy-succinimide-ester (FLUOS, Boehringer Mannheim). For three- and four-colour staining, biotinylated antibodies were detected with streptavidin–Cy-Chrome™ (Pharmingen, San Diego, CA) or streptavidin–allophycocyanin (Molecular Probes Europe, Leiden, The Netherlands).

In vivo labelling of thymocytes with BrdU

Mice were injected with a single intraperitoneal dose of BrdU (80 mg/kg, Sigma) dissolved in phosphate-buffered saline (PBS), and 4 h later thymi were removed and the single cell suspensions fixed in 70% ethanol. Immunostaining for BrdU combined with propidium iodide and FACS analysis were performed as described (Begg *et al.*, 1988). The anti-BrdU monoclonal antibody (ABDM) was purchased from CLB (Amsterdam, The Netherlands) and FITC-labelled goat anti-mouse antibody from Sigma.

ConA-stimulated primary T cell culture and BrdU labelling

To generate ConA blasts, single cell suspensions from spleens were plated out in RPMI medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol and 2 μg/ml of ConA (Sigma). To obtain synchronous cultures, cells were first stimulated for 72 h with ConA, washed extensively and starved for 14 h before addition of IL-2. The IL-2 used was X63-IL2 conditioned medium (gift from A.Strasser) at a final concentration of 1%. In the labelling experiments, BrdU was added to 50 μM at the same time as IL-2. Samples were taken at the indicated times, fixed immediately with 70% ethanol and stored at 4°C in the dark until analysis.

Western blot

Cell lysates (1×10^6 cell equivalents/lane) were immunoblotted using enhanced chemiluminescence (Amersham) for detection. Anti-p27 antibody (C-19) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and anti-actin antibody (C4) from Boehringer Mannheim. Autoradiograms were scanned by densitometry. The ratio of the intensity of the $p27^{Kip1}$ band to the corresponding actin band intensity is plotted as a function of time following IL-2 addition.

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